DISCRIMINATION OF *BACILLUS ANTHRACIS* FROM CLOSELY RELATED MICROORGANISMS BY ANALYSIS OF 16S AND 23S rRNA WITH OLIGONUCLEOTIDE MICROCHIPS

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FIELD OF THE INVENTION

Methods and compositions are provided for the detection of *Bacillus anthracis* from closely related microorganisms of the *B. cereus* group, and to distinguish and classify the *B. cereus* group. A customized, analytical oligonucleotide microchip incorporating 16S and 23S rRNA-targeted nucleic acid probes, is used for the detection of *B. anthracis* and discrimination.

BACKGROUND

Bacillus anthracis, the causative agent of the highly infectious disease anthrax, belongs to the *Bacillus cereus* group, which also contains six other closely related species: *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and *Bacillus medusa*. A variety of methods have been reported for the selective identification of *B. anthracis*. These include direct testing of bacterial DNA with specific probes, PCR amplification followed by an in-tube assay, PCR with subsequent electrophoretic analysis of length variation among ribosomal operons, ribotyping, amplified fragment length polymorphisms, methods of analysis using plasmid and chromosomal sequences, PCR-ELISA, on chip PCR amplification of anthrax toxin genes, detection of unique polysaccharides and other biomarkers on *B. anthracis* cell surface with mass spectrometry, immunological recognition of spores and vegetative cells and determination of phenotypic characteristics. The main goal of the various methods is

rapid and inexpensive detection of this extremely pathogenic microorganism so that containment, destruction of the pathogens and treatments are facilitated.

Hybridization analysis of 16S rRNA is a method of microbial identification. The 16S rRNA molecule is suitable for use as a target for microbial identification and detection. Although conserved in sequence overall, the 16S rRNAs exhibit significant sequence variation in some regions. These differences in 16S rRNA sequences provide the basis for the design of nucleic acid probes of varying specificity, ranging from probes targeting all living organisms, to group-specific and species-specific probes. Another advantage of using the rRNAs as a target is the fact that these molecules are naturally amplified within the cell. In general, rRNA represents about 80% of total nucleic acids in microbial cells, and thus is present in many hundreds and thousands of copies per cell. This natural amplification allows for direct detection of rRNA sequences without the need for intermediate amplification via PCR.

The main limitations of current hybridization techniques in general are that they are time consuming and limited in terms of the number of probes which can be analyzed simultaneously. Oligonucleotide microchip technology is a rapid and high throughput platform for nucleic acid hybridization reactions. Moreover, a universal mini-column (syringe-operated silica mini-column) for nucleic acid isolation, fractionation, fragmentation, fluorescent labeling, and purification, as well as an inexpensive, portable fluorescent analyzer for hybridization imaging was reported. Using the prototype mini-column, oligonucleotide microchip and portable imager, hybridization patterns from both microbial and human cells were detected in less than 60 minutes.

Current detection techniques for *B. anthracis* identification such as PCR, electrophoretic analysis, PCR-ELISA, and mass-spectrometry require a considerable amount of time, are expensive, and are generally limited by the number of probes analyzed. Moreover, some of these detection techniques are incapable of discriminating closely related isolates, especially isolates that are differentiated by as little as a single base change in DNA or RNA. In addition to being expensive and time-consuming, many of these methods are not portable. The present invention is designed to address many of the problems mentioned above.

SUMMARY OF THE INVENTION

A method for detecting a particular isolate of *B. cereus* subgroups, including *B. anthracis,* in a sample includes the steps of:

(a) Placing on a microchip oligonucleotide probes targeted to rRNA sequences that discriminate the *B. cereus* subgroups.

(b) Providing conditions for hybridization of the probes with rRNA from the sample.

(c) Analyzing hybridization signals in the microchip from which the particular isolate is detected.

The oligonucleotide probes on the microchip are directed to 16S rRNA or 23S rRNA of various *B. cereus* subgroups organisms. The rRNA samples are labeled with fluorescent dyes or radio isotopes, or immunological labels or immuno-chemical labels or gold particles and the like. The oligonucleotide probes, whose sequences are listed in Table 5, discriminate subgroups Anthracis, Cereus A, Cereus B, Thuringiensis A, Thuringiensis B, Mycoides A and Mycoides B.

An aspect of the invention is a microarray with oligonucleotide probes that bind to the target sequences designated:

Target Name	5'to 3' Target Sequence
c-ps1	GAGCGAATGGATTAAGAGCT
c-ps2	GAGCGAATGGATTgAGAGCT
c-ps3	AGCTTGCTCTTATGAAGTTA
c-ps4	AGCTTGCTCTcAaGAAGTTA
c-ps5	TGCTCTTATGAAGTTAGCGG
c-ps6	TGCTCTcAaGAAGTTAGCGG
c-ps7	CATTTTGAACCGCATGGTTC
c-ps8	CATTTTGAACtGCATGGTTC
c-ps9	CATTTTGAACCGCATGGTTC
c-ps10	CATTTTGcACCGCATGGTgC
c-ps11	AACCGCATGGTTCGAAATTG
c-ps12	cACCGCATGGTgCGAAATTc
c-ps13	ATGGTTCGAAATTGAAAGGC
c-ps14	ATGGTgCGAAATTcAAAGGC
c-ps15	GAAATTGAAAGGCGGCTTCG
c-ps16	GAAATTcAAAGGCGGCTTCG
c-ps17	CATCCTCTGACAACCCTAGA
c-ps18	CATCCTCTGAaAACCCTAGA
c-ps19	GCTTCTCCTTCGGGAGCAGA
c-ps20	GCTTCcCCTTCGGGgGCAGA
c-ps21	TTATCGTGAAGGCTGAGCTG
c-ps22	TTATCGTaAAGGCTGAGCTG

c-ps23	TGATACC-AATGGTATCAGTG
c-ps24	TGATACCgAATGGTATCAGTG

Lower case letters refer to positions of mismatches among the *B. cereus* subgroups (see FIGS. 1 and 2).

This invention also includes a microarray with oligonucleotide probes, whose sequences are designated:

Oligonucleotide Name	5' to 3' Sequence
ps1	AGC TCT TAA TCC ATT CGC TC
ps2	AGC TCT cAA TCC ATT CGC TC
ps3	TAA CTT CAT AAG AGC AAG CT
ps4	TAA CTT CtT gAG AGC AAG CT
ps5	CCG CTA ACT TCA TAA GAG CA
ps6	CCG CTA ACT TCt TgA GAG CA
ps7	GAA CCA TGC GGT TCA AAA TG
ps8	GAA CCA TGC aGT TCA AAA TG
ps9	GAA CCA TGC GGT TCA AAA TG
ps10	GcA CCA TGC GGT gCA AAA TG
ps11	CAA TTT CGA ACC ATG CGG TT
ps12	gAA TTT CGc ACC ATG CGG Tg
ps13	GCC TTT CAA TTT CGA ACC AT
ps14	GCC TTT gAA TTT CGc ACC AT
ps15	CGA AGC CGC CTT TCA ATT TC
ps16	CGA AGC CGC CTT TgA ATT TC
ps17	TCT AGG GTT GTC AGA GGA TG
ps18	TCT AGG GTT tTC AGA GGA TG
ps19	TCT GCT CCC GAA GGA GAA GC
ps20	TCT GCc CCC GAA GGg GAA GC
ps21	CAG CTC AGC CTT CAC GAT AA
ps22	CAG CTC AGC CTT tAC GAT AA
ps23	CAC TGA TAC CAT TG GTA TCA
ps24	CAC TGA TAC CAT TcG GTA TCA
ps25	CGGTCTTGCAGCTCTTTGTA
ps26	ATTCCAGCTTCACGCAGTC

Lower case letters refer to positions where mismatches are present in the target

sequences (see FIGS. 1 and 2).

This invention further includes an arrangement of the oligonucleotide probes in a microarray. The probes may be arranged in pairs. The pairs can be arranged in such a way that the presence or absence of a particular subgroup can be interpreted easily.For example, a customized microchip wherein I, II, III and IV are columns and A, B, C, D, E, and F are rows in the microchip design as follows:

	I	II	III	IV
A	ps19	ps20	ps7	ps8
B	ps15	ps16	ps3	ps4
C	ps9	ps10	ps5	ps6
D	ps13	ps14	ps1	ps2
E	ps11	ps12	-	-
F	-	-	ps17	ps18

This invention further includes an arrangement of the oligonucleotide probes as pairs in a microarray. The oligonucleotide probes are arranged in pairs in the following fashion: ps19 and ps20; ps15 and ps16; ps9 and ps10; ps13 and ps14; ps11 and ps12; ps7 and ps8; ps3 and ps4; ps5 and ps6; ps1 and ps2; ps17 and ps18. The pairs can be arranged in such a way that the presence or absence of a particular subgroup can be interpreted easily. One such arrangement is shown in FIG. 4.

A microarray represented in FIG. 8 is also an aspect of this invention.

Arrangement of the oligonucleotide probes as pairs in the microarray as in FIG. 8: 23F1 and 23F2; 23F5 and 23F6; 23F7 and 23F8; 16A1 and 16A2; 16A3 and 16A4; 16A5 and 16A6; 16A7 and 16A8; 16A9 and 16A10; #54 and SB25; SB10 and SB11; A7 and A8; 23F3 and 23F4; SB23 and SB22; D1 and D2A; B1 and B2; B7 and B8; C5 and C6; C7 and C8; A3 and A4; 23F13 and 23F14; 23F15 and 23F16; SB22 and SB23; B11 and B12; C9 and C10; C11 and C12; SB12 and #44; SB15 and SB16; SB4 and SB4-1; A1 and A2; A5 and A6; A9 and A10; A11 and A12. The pairs can be arranged in such a way that the presence or absence of a particular subgroup can be interpreted easily. A representative example of one such arrangement is shown in FIG. 8.

This invention includes a diagnostic kit to detect *B. anthracis* target rRNA in a sample. This kit includes in separate compartments:

(a) A microchip that comprises at least one oligonucleotide probe to

distinguish variations among *B. cereus* group isolates.

(b) Means for detecting hybridization between the probes and a target rRNA by which *B. anthracis* is detected.

This invention also includes a method for taxonomically classifying *B. cereus* group. This method includes the steps of:

(a) developing strain- and subgroup-specific signature profiles of 16S and 23S rRNA sequences for *B. cereus* group isolates; and

(b) using the signature profiles to construct phylogenetic trees in order to classify the various *B. cereus* group isolates.

This invention includes a microarray with oligonucleotide probes, whose sequences are listed in Table 5.

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Array, microarray: molecules connected to the matrix or support in a specific arrangement relative to each other.

Biochip: also known as a chip, DNA chip, DNA microarray, DNA array, microchip, peptide chip or peptide array; includes array of biological molecules such as DNA fragments, peptides, proteins, lipids, and tissues connected to a matrix.

Biological sample: a biological material obtained from blood, liver, skin, tissues, saliva, tears, bodily fluids or bodily secretions.

Isolate: a particular genetic variant of a species. If one isolate is known, then it defines the species. However, there can be many different isolates of one species, isolated for example, from different patients or different parts of the world.

Sample: includes biological samples such as blood, skin, bodily fluids and tissues and environmental samples such as air, food, water and soil.

Placing on a microchip: refers to a process by which oligonucleotides are attached to a microarray.

Providing conditions for hybridization: refers to experimental setup that includes as appropriate buffers, temperature, and time that are essential for hybridization of nucleic acids.

Analyzing hybridization signals: a method of detecting and interpreting

Signature profiles: a compilation of mismatches of nucleotide sequence that is specific for a particular strain or subgroup of microorganisms.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the positions of subgroup–specific sequence differences in the 16S rRNA gene of *B. cereus* subgroups (A) and reference microorganisms used for microchip testing (B). The sequence of *B. anthracis Ames ANR* was used as the consensus sequence. Sequences c-ps1 through c-ps20 which are complementary to the probes ps1 through ps20 on a microchip (see page 3-5, 52-53) and their locations on the 16S rRNA are also shown (bold letters denote target nucleotides). The names of the target sequences (example, c-ps1) are shown to the left of each of the sequences and the corresponding probe sequences (example, SB1) are shown to the right of the sequences. The probe sequences (ps1-ps20) for the target sequences are listed in Table 5.

FIG. 2 illustrates the positions of subgroup–specific sequence differences in the 23S rRNA. The sequence of *B. anthracis Ames ANR* was used as the consensus sequence. Arrows indicate regions containing subgroup-specific signatures. Target sequences (c-ps21 through c-ps24) complementary to the probes (ps21 through ps24) and their locations on the 23S rRNA are also shown (bold letters denote target nucleotides). The corresponding probes sequences (example, ps21) are listed in Table 5. R =G, or A; Y = T, or C.

FIG. 3 illustrates the phylogenetic trees of (A) 16S and (B) 23S rRNA genes of bacteria in *B. cereus* group. Sequence analysis was performed using the multiple sequence alignment computer programs "Clustalx" (ftp://ftp-igbmc.u-strausbg.fr/pub/clustalx/), and "Clustlaw" (http://www.ebi.ac.uk/clustlaw). Asterisks indicate the reference microorganisms which were used.

FIG. 4 illustrates the identification of reference microorganisms and subgroups to which they belong in the *B. cereus* group with a 16S rRNA oligonucleotide microchip. Total RNA from reference microorganisms was isolated, fluorescently labeled with Texas Red, and hybridized with a microchip bearing 20 bases of oligonucleotides as described in MATERIALS AND METHODS. Positions of the probes and targeted subgroups (in rectangles) are indicated in the upper left corner. Members of the targeted subgroup form perfect matches with probes indicated with arrows. For probe abbreviations see FIG.

FIG. 5 illustrates the identification of single-base polymorphisms (A) and differentiation of Cereus A subgroup bacteria (*B. thuringiensis* B8 and *B. cereus* 1414) from organisms of Anthracis subgroup (*B. anthracis* Ames) (B), using hybridization of fluorescently labeled total RNA from *B. cereus* group bacteria to probes targeting the 23S rRNA. R = G, or A. Probe signal ratio represents an average from 2-4 experiments.

FIG. 6 illustrates the identification of 16S rRNA of (A) *B. anthracis* Ames in a mixture (3:2) with *B. cereus* NCTC9620 16S rRNA and (B) *B. thuringiensis* B8 (*B. anthracis* mimic) 16S rRNA mixed with *B. thuringiensis* 4Q281 16S rRNA in the ratio 1:10. Total RNA of the studied bacteria was isolated, fluorescently labeled as described in MATERIALS AND METHODS, mixed in the above mentioned proportions, and hybridized with an oligonucleotide microchip. For probe abbreviations shown on the left and right sides of panels, see FIG. 1. Bold numbers indicate the ratio of integrated fluorescent signals after hybridization.

FIG. 7 illustrates the identification of microbial groups using a 16S rRNA oligonucleotide microchip. A microchip containing oligonucleotides ps25 and ps26 targeting the *B. cereus* group (5'-CGGTCTTGCAGCTCTTTGTA-3') and the *B. subtilis* group (5'-ATTCCAGCTTCACGCAGTC-3'), respectively is shown. Microchips were hybridized with fluorescently labeled total RNA of the corresponding microorganisms. Ratios of integrated fluorescent signals are shown in the far right column.

FIG. 8 is a map of a microchip with oligonucleotide probes (for example, 23F1) whose sequences and target names (for example, Mycoides B) are listed in Table 5. The positions of oligonucleotides are designated by squares and the brief description inside the square indicates name of the oligonucleotide probe and the targeted subgroups.

DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions are presented for using nucleotide sequence variations of 16S and 23S rRNA within the *B. cereus* group to discriminate *B. anthracis* from closely related microorganisms. The existence of sequence variability within the *B. cereus* group was useful to consistently determine the identity of *B. cereus* isolates including *B. anthracis*. To accomplish this goal a set of 16S and 23S

rRNA targeted oligonucleotide probes was designed to discriminate among the seven subgroups within *B. cereus*, and in particular to discriminate *B. anthracis* from other members in the *B. cereus* group. The sequences for these probes were selected so that they are complementary to target rRNA sequences. These probes were incorporated into an oligonucleotide microchip. Feasibility of discrimination of single base differences in rRNA was demonstrated with this microchip during analysis of *B. cereus* group isolates from both single and mixed cultures. Rapid, selective identification of *B. anthracis* from a mixture of closely related microorganisms has valuable application in diagnosis and epidemiological monitoring.

Aspects of the invention include:

1. microchips designed to detect a particular isolate of *B. cereus* subgroup, including *B. anthracis* based on sequence variations in the 16S and 23S rRNAs; the sequence variations are chosen so that hybridization signals discriminate a particular isolate from other closely related organisms; using at least one mismatched sequence; and
2. methods for improved taxonomic classification and detection of *B. cereus* group isolates based on individual strain variations of 16S and 23S rRNA sequences.

The microchips of the present invention overcome some of the limitations of current hybridization techniques to detect *B. anthracis*. The microchip-based detection of variations in rRNA sequences is rapid, reliable, and capable of high throughput. Additionally, small sequence variations such as single nucleotide polymorphisms (SNPs) among closely related isolates can be effectively discriminated with the microchip disclosed in the present invention. Because rRNA-based hybridization does not require PCR amplification, a direct and efficient method of detection is possible with microchips. Hybridization signals can be analyzed by an inexpensive fluorescent analyzer, which is also portable. This portability coupled with the ease of detecting *B. anthracis*, a highly infectious agent renders the current invention a valuable tool for public health safety measures.

The present invention also discloses variations of 16S and 23S rRNA sequences among *B. cereus* isolates. These sequence variations are essential to correctly classify closely related microorganisms. Analysis of 16S and 23S rRNA sequence variations in *B. cereus* group isolates revealed certain subgroup- and strain-

specific signatures that aid in the grouping of closely related isolates. Correct classification of these isolates is important to identify the close relationships and to develop better analytical methods to discriminate among the isolates. For example, appropriate clustering of subgroup-specific sequence variants of the present invention provides the basis for the design of a number of diagnostic oligonucleotide probes to discriminate each of the subgroups within the *B. cereus* group.

Diagnostic kits to discriminate *B. anthracis* from closely related microorganisms include:

(a) At least one microchip that includes at least one oligonucleotide probe that is discriminating, usually distinguishing among related organisms by at least one mismatch between target rRNA sequences. Suitable probes are those in Table 5;

(b) Means for detecting hybridization signals between labeled RNA and oligonucleotides on the microchip.

Means for detecting hybridization signals include a fluorescence microscope equipped with a CCD camera or a laser scanner. Reagents for isolating total RNA include nucleic-acid spin columns (Bavykin et al., 2001) and GITC-based RNA extraction reagents. Fluorescent dyes such as LissRhod (Lissamine™ rhodamine B ethylenediamine; Cat # L2424 and Texas Red cadaverine; Cat # T2425 (Molecular Probes Eugene, OR) can be used to label rRNA molecules isolated from microorganisms.

Customized oligonucleotide microchips are aspects of the invention. The microchip includes a matrix support, which can be made from elements such as glass, and polyacrylamide. An embodiment of a microchip is:

(a) ten pairs of oligonucleotide probes that target 16S rRNA sequences and two pairs of oligonucleotide probes targeting 23S rRNA sequences; the oligonucleotides are synthesized to include a 5'-amino-modifier;

(b) microchips containing polyacrylamide gel pads with aldehyde groups; and

(c) one to six nl of individual amino-oligonucleotide solutions in each gel pad element.

An embodiment of a customized microchip includes an array wherein

oligonucleotides are arranged in a specific pattern as in FIG. 8 and sequences of the oligonucleotides are selected from Table 5. Another embodiment of a customized microchip is an array wherein oligonucleotide probes are immobilized in a specific pattern as in FIG. 4 and the probe sequences are selected from Table 1 (ps1 through ps20). Using these customized microchips, *B. anthracis* can be discriminated from other closely related isolates.

Identification of subgroups and strains in *B. cereus* group with rRNA probes

Based on 16S rRNA sequence analysis, the *B. cereus* group was divided into seven subgroups (Anthracis, Cereus A and B, Thuringiensis A and B and Mycoides A and B) each containing microorganisms with similar 16S rRNA gene sequences (FIG. 3A and Table 2). The strains within each subgroup contained all of the sequence variants specific for that subgroup. The 23S rRNA sequencing confirmed these subgroup classifications (FIG. 3B and Table 3). Some of the subgroup-specific signatures, indicated in Table 2, have already been reported for identification of certain *Bacillus* strains. For example, *B. thuringiensis* was differentiated from *B. cereus* based on a sequence difference in region 77-92. However, only isolates from subgroup Thuringiensis B, and not isolates from subgroup Thuringiensis A (FIG. 1 and Table 2), could be differentiated based on this sequence difference. Psychrotolerant strains of *B. cereus* or *B. mycoides* have been identified based on differences in regions 182-197 and 1019-1030 of their 16S rRNA sequences, but these signatures describe organisms from subgroup *mycoides A* only, and not isolates from subgroup *mycoides B* (FIG. 1 and Table 2). Therefore, systematic analysis of all *B. cereus* group microorganisms had not been done.

A set of 16S rRNA targeted oligonucleotide probes (FIG. 1) is constructed for use in 3-D gel pads. These probes were immobilized within a oligonucleotide microchip. This microchip enabled differentiation of *B. anthracis* Ames (subgroup Anthracis) and *B. thuringiensis* B8 (subgroup Cereus A) from six reference strains of closely related organisms (*B. cereus* T, *B. thuringiensis* 4Q281, *B. medusa* ATCC25621, *B. mycoides* ATCC 6462m, *B. mycoides* ATCC 10206. *B. cereus* 9620) representing three different subgroups, Cereus B, Thuringiensis B and Mycoides B, respectively. An embodiment of the present invention is a customized microchip capable of identifying organisms of subgroups Thuringiensis A. Comparison of

hybridization signals from probe pairs ps1/ps2, ps3/ps4 and ps5/ps6 with signals from pair ps7/ps8 on the microchip (FIG. 1) demonstrated that *B. thuringiensis* str. 4W1, 4T1, 4F1 and 4D1 belong to subgroup Thuringiensis A, whereas *B. thuringiensis* str.4Q1, 4Q2, 4A1 and 4A7 belong to subgroup Thuringiensis B.

Because the RNA sequences of bacteria from the Mycoides A subgroup became available only recently, specific probes for this subgroup are present only n a microarray as shown in FIG. 8 and not as shown in FIG. 4. However, the microchip, whose configuration is disclosed in FIG. 4 also has the capability to recognize organisms of Mycoides A subgroup. Results of microchip hybridization is similar with subgroup Thuringiensis A organisms, but signals from pairs ps7/ps8, ps9/ps10, and ps11/ps12 (FIG. 1) are considerably decreased in comparison with that one for Thuringiensis A, because of the forming of two additional mismatches for ps7/ps8 and ps9/ps10, and one additional mismatch in the middle of the probes, ps11/ps12. Signals from the probes ps13/ps14 and ps17/18 may be also decreased because of the presence of one additional mismatch in the terminal end of these probes. However, discrimination is easily achieved using regions 120-145, 166-188, 1015-1035 on 16S rRNA and region 366-390 on the 23S rRNA genes as probe targets. Probes for these regions have also been selected and applied on the second generation of microchips for identification f *B. cereus* group microorganisms (FIG. 8, Table 5).

Another embodiment of the present invention is a method for differentiating microbial strains that differ by only one base in their 16S rRNA molecule both separately (FIG. 4) and in mixtures (FIG. 6). Thus it is possible to identify all strains within the *B. cereus* group that differ by as little as a single nucleotide change in their rRNA sequences. Based on 16S rRNA sequence differences (Table 2), the microchip also serves to differentiate isolates of subgroup Anthracis and subgroup Cereus A from all other thirty-two studied strains of bacteria in the *B. cereus* group and to identify which subgroup (Table 2) each microorganism belongs (FIG. 4), even in a 1:10 mixture (FIG. 6).

Another embodiment of the present invention is to be able to identify *B. cereus* isolates by the sequence variations in their 23S rRNA. The 23S rRNA gene was sequenced for a selected set of reference strains of the *B. cereus* group. Isolates from subgroup Cereus A, which has the same 16S rRNA sequence as *B. anthracis* Ames (FIG. 1 and Table 2), have three changes in the 23S rRNA sequence in

comparison with *B. anthracis* Ames (FIG. 2 and Table 3). *B. thuringiensis* B8 and *B. cereus* HER1414 were used to demonstrate that these sites may be utilized for discrimination between subgroups Anthracis and Cereus A (FIG.5).

Study of site 1559 on the 23S rRNA, where a number of strains revealed single-base changes, demonstrated that the microchip also enabled a single-base polymorphism to be detected (FIG. 5A).

Previous work has shown that 16S rRNA sequences for *B. anthracis* Sterne (subgroup Anthracis), as well as *B. cereus* NCDO1771 and *B. cereus* NCTC 11143 (subgroup Cereus A) have 99.9-100% similarity (Table 1). However, all the organisms that belong to Cereus A and Anthracis subgroups, are differentiated using subgroup-specific signatures, or strain-specific variations and a combination of 16S and 23S rRNA-targeted probes (Tables 2 and 3). False negative identifications, are not expected *i.e.* the methods will effectively recognize the presence of *B. anthracis*. Very seldom, some false positive identifications occurred, *i.e.* mistaken identification of *B. anthracis* strains that lost one or both of their plasmids, and therefore, lost their virulence as *B. anthracis*. However, for the identification of a species, which produces a toxin as hazardous as anthrax, a small number of false positive reactions is preferable to any false negative signals.

Therefore, the microchips (FIG. 4 and FIG. 8) is capable of discriminating all seven subgroups of the *B. cereus* group, and thus microchip analysis of ribosomal RNA serves as a powerful tool for identification of *B. cereus* group bacteria.

Taxonomy of the *B. cereus* group

The results of analysis of 16S and 23S rRNA sequences show some disagreement with the current taxonomic classification of species within the *B. cereus* group. Traditionally, classification of microorganisms in the *B. cereus* group has been based on morphological, physiological, and immunological data. However, some data suggests that there may be some difficulties with these classification schemes. *B. thuringiensis* has been traditionally distinguished from *B. cereus* by the production of a parasporal crystal of a protein that is toxic for Lepidoptera, Diptera and Coleoptera larvae. The capacity to form crystals is plasmid-encoded, however, the plasmid may be lost by laboratory culturing. Moreover, authentic cultures of *B. cereus* can acquire the ability to produce crystals as a result of growing in mixed

culture with *B. thuringiensis*. Thus, the discrimination of *B. cereus* from *B. thuringiensis* is a difficult task by any method, and the fact that they have grouped together in the present analysis is not surprising. At the same time, some *B. thuringiensis* strains may be moved (reassigned) after resequencing their 16S rRNA from subgroup Cereus B to subgroup Thuringiensis B, which differ from each other by only one subgroup-specific signature C/T(192) (Table 2).

Sporadic loss of the ability to form rhizoid colonies has been observed in several strains of *B. mycoides*, indicating an instability of morphology in this species. DNA relatedness studies have indicated that the species *B. mycoides* actually consists of two genetically distinct groups. The fact that methods and compositions of the present invention place *B. mycoides* strains into two subgroups, Mycoides A and Mycoides B, supports this finding. Bacterial strains can also undergo physiological changes after the loss or acquisition of plasmids coding for toxins, sporulation, or antibiotic resistance.

According to the present classification scheme (Table 2), four representatives of psychrotolerant strains of *B. cereus* (WSBC10201, WSBC10204, WSBC10206 and WSBC10210), which were recently named as the new species *B. weihenstephanensis*, fall under subgroup Mycoides A. This finding suggests that species *B. weihenstephanensis* may be one of the *B. mycoides* strains that belongs to the subgroup Mycoides A. This suggestion is confirmed by the high degree of similarity of genomic DNA sequences (85-88%) between *B. cereus* strains WSBC10201, WSBC10204, and WSBC10206 and *B. mycoides* DSM2048, which is also located in subgroup Mycoides A. In addition, based on the ability to grow at low temperature, *B. mycoides* is the most closely related species to *B. weihenstephanensis* in the *B. cereus* group.

EXAMPLES

Example 1: Sequencing of 16S and 23S rRNA genes of *B. cereus* group microorganisms

Twelve 16S rRNA and ten 23S rRNA genes were sequenced (Tables 2, 3). There are published sequences available for two of the strains that were sequenced, *B. medusa* NCIMB 10437 (ATCC 25621) and *B. anthracis* Sterne. There were some discrepancies between the present sequences and the previously published sequences.

The published 16S rRNA sequences of *B. anthracis* Sterne (GenBank AC: X55059) and *B. medusa* NCIMB 10437 (GenBank AC X60628) have a deletion of C (942) in comparison with other strains of *B. anthracis* and *B. medusa* that were sequenced later (Table 2). This deletion was found neither in the present resequencing of *B. anthracis* Sterne (GenBank AC: AF176321) and *B. medusa* ATCC25621 (GenBank AC AF155958), nor in the present and in TIGR sequencing of *B. anthracis* Ames (GenBank AC:AF267734 and website http://www.tigr.org, respectively). It is likely that the reported deletion was a compression artifact of sequencing of the GC-rich region, i.e. -GGGGCCG- instead of -GGGGCCCG-. The same compression artifact may also have compromised the 16S rRNA sequences of *B. cereus* NCDO 1771, *B. cereus* NCTC 11143, *B. mycoides* DSM 2048 and *B. thuringiensis* NCIMB 9134 (GenBank AC:X55060 to X55063).

In addition, resequencing of the 16S rRNA gene for *B. medusa* ATCC 25621, did not reveal the C to T transition at position 192 (presence of T instead of C found in *B. anthracis*), or the A to G transversion at position 1383 previously reported for *B. medusa* NCIMB 10437 16S rRNA (Table 2).

Differences were also found in the previously published 23S rRNA sequence of *B. anthracis* Sterne (GenBank AC: S43426) and the present resequencing of this strain (GenBank AC: AT267877). The differences found were the following: T instead of C in position 491, deletion of CG(1413, 1414), and T instead of C in position 2651. These changes were not found in any other 23S rRNA sequence in *B. cereus* group, including *B. anthracis* Ames and *B. anthracis* DeltaAmes (Table 3). Therefore, it is likely that these differences in *B. anthracis* Sterne and also the same differences in *B. cereus* 11143 (GenBank AC X64646) are due to errors in the previously reported sequence.

Example 2: Comparison of 16S and 23S rRNA sequences in the *B. cereus* group

The present analysis indicated that in terms of known 16S and 23S rRNA sequences, *B. anthracis* was the most homogeneous species within the *B. cereus* group. This finding confirms PCR fingerprinting studies that demonstrated almost complete homogeneity of *B. anthracis* bulk DNA. In this work no reliably established variation in the 16S or 23S rRNA sequences was observed within any of the five *B. anthracis* strains characterized (Tables 2 and 3, FIGs. 1 and 2). Because of

this homogeneity, and because *B. anthracis* is a target organism for the present invention, the *B. anthracis* 16S and 23S rRNA sequences were used as a reference for reporting differences among closely related bacteria within the *B. cereus* group (FIGs.1 and 2, Tables 2 and 3).

The present analysis of 16S rRNA sequences for the other *B. cereus* group organisms identified six characteristic regions which contained the majority of the sequence differences among members of the groups: position(s) 77-92 , 133 , 182-208, 286, 1015-1045 and 1464 (FIG.1 and Table 2). Because sequence variation in these regions can be used to divide the *B. cereus* group organisms into several large subgroups, the differences located within these regions were termed subgroup-specific signatures. Eighty percent of the strains of *B. cereus*, *B. thuringiensis*, *B. medusa*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (32 of 40 sequences) analyzed contained some subgroup-specific signatures (Table 2) in their 16S rRNA sequences. The most common were C/A (1015) and C/T (192). In addition, a number of other differences were observed, which were termed strain-specific signatures (Table 2). These strain-specific signatures were unique to each strain and were located randomly along the 16S rRNA molecule, i.e., they did occur within the same sites as the subgroup-specific alterations. *B. anthracis* differed from 37 of the 40 other organisms within the *B. cereus* group by at least one sequence difference in the 16S rRNA.

Analysis of the 23S rRNA sequences for the *B. cereus* group organisms revealed thirteen regions within which the majority of the sequence variation occurred (FIG. 2 and Table 3). The differences within these regions are analogous to the subgroup-specific signatures found in the 16S rRNA.

However, due to the limited number of 23S rRNA sequences in the GenBank database, it may be that not all of these differences are subgroup specific. Some of the regions, which appear to contain subgroup-specific variants, may actually contain only strain-specific regions. For example, the Mycoides B subgroup showed five subgroup-specific differences in the 16S rRNA and eleven subgroup-specific differences in 23S rRNA sequences that were not found in other subgroups (FIG. 2). However, available rRNA sequences for the Mycoides B subgroup currently contain only four strains for which 16S rRNA sequences were determined and two strains for 23S rRNA sequences (Table 2, 3). Among them, *B. mycoides* ATCC6462m and *B.*

mycoides ATCC10206, have identical 16S and 23S rRNA sequences (FIG. 1 and 2), as well as 16S-23S rRNA spacer, however differed with their colony morphology. If additional members of the Mycoides B subgroup are sequenced and added to the GenBank database, some of the subgroup-specific signatures may be actually strain-specific.

Both the 16S and 23S rRNA sequence sets showed alterations, which were present in a majority of the subgroups. Subgroups Cereus B, Thuringiensis A, Thuringiensis B, and Mycoides A all contained a C/A difference at position 1015 in their 16S rRNA sequences. The most common subgroup specific differences in 23S rRNA sequence occurred at positions 157 and 594 (Table 3, FIG. 2). The presence of these common variants among the subgroups supports a phylogenetic relationship among them.

Example 3: Grouping of microorganisms in *B. cereus* group according to 16S rRNA sequences

The *B. cereus* group can be divided into seven subgroups based on 16S rRNA sequence differences (Table 2). Each of these subgroups were identified according to the name of the most common member of the subgroup: Anthracis, Cereus A and B, Thuringiensis A and B, and Mycoides A and B. Based on 16S rRNA sequences, an unrooted phylogenetic tree was also inferred for the *B. cereus* group using the computer program "Clustalx" (FIG. 3A). Although the affiliations in the tree are generally consistent with those defined by signature analysis (Table 2), these groupings do not correspond exactly to the current taxonomy, which divides the *B. cereus* group into seven species: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. medusa*, *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis*.

The following subgroups were described according to the 16S rRNA sequences (Table 2):

Subgroup Anthracis includes five strains of *B. anthracis*. These organisms do not contain any reliably established subgroup-specific or strain-specific sequence differences in comparison with the *B. anthracis* consensus sequence. Subgroup Cereus A includes eight members, which do not contain any subgroup-specific sequence differences from the *B. anthracis* consensus sequence, however were not classified as *B. anthracis* by conventional taxonomic methods. Of these strains, four were found to contain strain-specific sequence differences in their 16S rRNA

sequences. However, three of other four strains, B. sp. strain JJ#1, *B. cereus* NCTC11143, and *B. thuringiensis* B8, were found to have sequences identical to subgroup Anthracis in the region of the 16S rRNA compared (about 100 nucleotides at the 3'-end of the 16S rRNA have not yet been sequenced for two of these three strains (Table 2)). Two strains of the subgroup Cereus A, *B. cereus* WSBC10037 and *B. cereus* 10030, have been characterized as mesophilic. As the result of the present invention, *B. cereus* HER1414, whose 16S rRNA genes are not yet sequenced, was also included in cereus A subgroup on the basis of hybridization with the microchip represented on FIG. 5B.

Subgroup Cereus B includes strains of *B. cereus* and *B. thuringiensis* that differ from *B. anthracis* by a C to A change at position 1015. The strains *B. cereus* NCTC9620, *B. cereus* T, *B. cereus* IAM12605, also named *B. cereus* 1771, and *B. thuringiensis* WS2626 do not differ from one another in the 16S rRNA sequence, and thus they would be indistinguishable based on 16S rRNA hybridization.

Subgroups Thuringiensis A and Thuringiensis B include strains which contain two and five subgroup-specific sequence differences respectively, C/A (1015) and C/T (192) being shared among the two subgroups. These two subgroups include mainly *B. thuringiensis* strains. Two strains in the subgroup Thuringiensis B (*B. thuringiensis* 4Q281 and *B. thuringiensis* IAM12077 which was also named *B. thuringiensis* NCIM9134 or *B. thuringiensis* OSM2046) have identical 16S rRNA sequences. Two other strains within this subgroup, *B. medusa* ATCC25621 and *B. medusa* NCIMB10437, should be identical according to Bergey's Manual. However, according to sequencing (Table 2) and hybridization studies (FIG. 4), strain *B. medusa* ATCC25621 does not contain the subgroup-specific signature C/T(192), whereas according to published sequences, *B. medusa* NCIMB10437 does contain this sequence variant.

In the last two subgroups, Mycoides A and Mycoides B, five *B. mycoides* strains group in subgroup Mycoides A, and four fall under subgroup Mycoides B. Psychrotolerant strains *B. weihenstephanensis* DSM11821 and *B. cereus* strs. WSBC 10201, 10204, 10206 and 10210, which have been characterized as *B. weihenstephanensis*, were also included in subgroup Mycoides A. Subgroup Mycoides B contains *B. cereus* ki21 and *B. pseudomycoides*, which may have split off from the other two isolates (*B. mycoides* ATCC-10206 and *B. mycoides* ATCC

6462m) in this subgroup rather early in their evolution, as they have a large number of strain-specific sequence differences (Table 2).

Example 4: Grouping of microorganisms in *B. cereus* group according to 23S rRNA sequences

Based on a similar analysis of 23S rRNA sequences (FIG. 2) the *B. cereus* group strains could be divided into six subgroups. These divisions were consistent with the phylogenetic tree inferred using the computer program "Clustalx" (FIG. 3B). These 23S-based subgroups correspond to six of the seven 16S-based subgroups, namely Anthracis, Cereus A and B, Thuringiensis B and Mycoides A and B (Tables 2 and 3). There were no 23S rRNA sequences available for any of the organisms from the subgroup Thuringiensis A. Therefore, based on the available data, division of the *B. cereus* group members into the specified subgroups is supported by both the 16S and 23S rRNA. Also, as with the 16S rRNA sequences, all *B. anthracis* strains had identical 23S rRNA sequences.

Subgroup Cereus A (Table 3) contains *B. thuringiensis* B8 and *B. cereus* NCTC11143. The 23S rRNA sequences of these two organisms include alterations at positions 594, 1559 and an insertion G(1219) (FIG. 2 and Table 3). The third member of the subgroup, *B. cereus* HER1414, whose rRNA operon is not sequenced yet, contains subgroup-specific signatures in positions 1218 and 1559. These signatures were found in the hybridization experiments disclosed herein. The third subgroup-specific signature, at position 594, was not tested for this strain. The 23S rRNA sequence of *B. cereus* WSBC10030 was sequenced only partially (FIG. 2, Table 3). It does not contain any signatures that are specific for any other subgroups, but also does not cover subgroup-specific sites for Cereus A subgroup. For this reason *B. cereus* WSBC10030 (Table 2 and 3) and other five strains, *B. cereus* strs. BSID723, WSBC10037, 1396 and B. sp.JJ#1 (Table 2) were placed into Cereus A subgroup conditionally. Final discrimination of these isolates from Anthracis subgroup bacteria awaits testing of the 23S rRNA sequences of these microorganisms.

B. cereus T, *B. cereus* NCTC9620, *B. cereus* DSM31 (also named *B. cereus* IAMI2605, *B. cereus* NCD01771) *B. thuringiensis* WS2614, and *B. thuringiensis* WS2617 were placed in subgroup Cereus B based on both the 16S and 23S sequence comparisons. Unfortunately, 23S rRNA sequences available for both *B. thuringiensis* strains do not extend beyond position 527 from the 5'-end of the gene.

Subgroup Thuringiensis B consists of three strains, *B. thuringiensis* 4Q281, *B. medusa* ATCC25621 and *B. thuringiensis* DSM2046 (also named *B. thuringiensis* IAMI207 or *B. thuringiensis* NCM9134). Sequences of the 16S rRNAs are available for all three bacteria, (Table 2). A finding from the 23S rRNA sequence analysis was the relationship between subgroups Thuringiensis B and Mycoides B. According to the 16S rRNA sequences, subgroups Thuringiensis B and Mycoides B do not share any subgroup-specific sequence signatures (FIG. 1, Table 2). However, subgroups Thuringiensis B and Mycoides B do share eight positional variants in their 23S rRNA sequences (FIG. 2, Table 3). This 23S rRNA data suggests that the organisms in these two groups may be phylogenetically related (FIG. 3B).

B. mycoides 2048T, *B. mycoides* MWS5303-1-4 and *B. cereus* WSBC10206 according to their 16S rRNA sequences belongs to subgroup Mycoides A. Unfortunately, only 527 nucleotides of sequence from the 5'-end of these 23S rRNAs are available for the last two organisms, however, they revealed enough variations to be discriminated from members of all other subgroups (Table 3, FIG. 2).

The clustering of subgroup-specific sequence variants demonstrated by the present invention provided the basis for the design of a number of diagnostic oligonucleotide probes for the identification of each of the different subgroups within the *B. cereus* group. In addition, some of the sequence variants were useful for the design of probes for the identification of individual strains.

Example 5: Design of subgroup-specific probes

Results of the 16S and 23S rRNA gene sequencing indicated that microorganism identification within the *B. cereus* group would require single base mismatch discrimination. The optimal probe length was determined, taking into account the increased efficiency of mismatch discrimination which results from decreased probe length, and the increased specificity of hybridization, duplex stability, and hence hybridization signal intensity, which results from longer probes. Computer analysis of rRNA sequences available from the Ribosomal Data Project website (http://www.cme.msu.edu/RDP) indicated that 10-15 base long probes would not provide adequate specificity, the optimum probe length was determined to be 17-23 bases long.

The sequences complementary to the probes, which were selected are shown

in FIGs. 1 and 2 and Table 5. The degree of matching between the probes and the rRNA sequences of the eight reference microorganisms is shown in Table 4. The location of the 16S rRNA probes on the microchip is shown in FIG. 4 and FIG. 5. The microchip on FIG. 4 includes three pairs of probes for identification of bacteria from subgroup Thuringiensis B (ps1/ps2, ps3/ps4 and ps5/ps6), one pair for identification of organisms from subgroups Thuringiensis A and B, five pairs for identification of Mycoides B subgroup bacteria (ps9/ps10, ps11/ps12, ps13/ps14, ps15/ps16 and ps19/ps20), and one pair for discrimination of subgroups Anthracis, Cereus A, and Mycoides B from subgroups Cereus B and Thuringienses A, B (ps17/ps18). The combined application of these probes provides a basis for distinguishing subgroup Anthracis from all other subgroups except Cereus A. Most bacteria of subgroup Cereus A revealed strain-specific sequence variation. Although some isolates in this subgroup have 16S rRNA sequences identical to *B. anthracis* (Table 2), these organisms do differ from *B. anthracis* in 23S rRNA sequences (FIG. 2). Therefore, probe pairs designed were ps 21/ps22 and ps23/ps24 (FIG. 2) for the discrimination of subgroups Anthracis and Cereus A using a sequence difference located respectively at positions 1559 and 1219 in the 23S rRNA molecule (Table 3).

Example 6: Identification strategy for subgroups and reference microorganisms

Eight reference organisms were selected (*B. anthracis* Ames, *B. thuringiensis* B8, *B. cereus* T, *B. cereus* 9620, *B. thuringiensis* 4Q281, *B. medusa* 25621, *B. mycoides* 6462m and *B. mycoides* 10206) to demonstrate the ability of microchips to differentiate subgroups Anthracis, Cereus A and B, Thuringiensis B and Mycoides B. Objectives were to determine if organisms from these closely related subgroups could be discriminated (as determined by 16S and 23S rRNA analysis), to determine if bacteria whose rRNA sequences differed by only one base could be discriminated, and to determine if *B. anthracis* could be discriminated from closely related species in the *B. cereus* group. Results of hybridization of LissRhod labeled total RNA of the reference bacteria with a microchip containing selected probes are shown in FIG. 4 and FIG. 5B.

Example 7: Identification of *B. mycoides* ATCC 6462m and *B. mycoides* ATCC 10206 (subgroup Mycoides B)

The results from microchip hybridizations (FIG. 4) were identical for these

two references *B. myocoides* strains. These results demonstrate the reproducibility of the microchip technique for characterizing independent isolates having identical rRNA sequences. The 16S rRNAs of *B. mycoides* 6462m and 10206 form perfect duplexes with probes ps10, ps12, ps14, ps16 and ps20 (set #1) but include mismatches with probes ps7(ps9), ps11, ps13, ps15 and ps19 (set #2). All other reference bacteria contain mismatches for set #1 probes and form perfect matches with set #2, except *B. thuringiensis* 4Q281 (subgroup Thuringiensis B), as well as organisms of Thuringiensis A subgroups, which contain one mismatch for probes ps7(ps9) and ps11 (FIG.1 and Table 4). Microorganisms that belong to subgroup Mycoides A contain two mismatches with probes ps9 and ps11 and one mismatch with probe ps13 (FIG. 1).

Example 8: Identification of *B. thuringiensis* 4Q281 (subgroup Thuringiensis B)

Probe pair ps7(ps9)/ps8 is specific for subgroups Thuringiensis A, B (FIG. 1). Probe ps8 forms a perfect match with *B. thuringiensis* 4Q281 16S rRNA and contains one or three mismatches for 16S rRNAs of all other reference bacteria. In contrast, probe ps7(ps9) contains a mismatch for *B. thuringiensis* 4Q281, and forms perfect matches with all other reference bacteria except *B. mycoides* (strains 6462m and 10206), which have two mismatches with probe ps7 (FIG.1 and Table 4). Organisms from subgroup Mycoides A have three mismatches with probe ps7(ps9) and two mismatches with probe ps8. Three probes from set #2, ps13, ps15 and ps19, form perfect match with 16S rRNA of *B. thuringiensis* 4Q281. In set #1 probe ps16 contains one mismatch, as well as probes ps14 and ps20 contain two mismatches for this bacteria.

Example 9: Differentiation of *B. medusa* ATCC 25621 and *B. thuringiensis* 4Q281

Probe pairs ps1/ps2, ps3/ps4 and ps5/ps6 are subgroup-specific for subgroup Thuringiensis B (FIG.1, Table 4). Probe set #3 (ps2, ps4, and ps6) forms perfect duplexes with the 16S rRNA of *B. medusa* ATCC 25621 and *B. thuringiensis* 4Q281 and mismatches with 16S rRNAs of all other reference microorganisms. In contrast, probe set #4 (ps1, ps3 and ps5) forms mismatches with RNA isolated from *B. medusa* 25621 and *B. thuringiensis* 4Q281 and perfect matches with 16S rRNA of all five other reference bacteria. *B. medusa* 25621 and *B. thuringiensis* 4Q281 can be

discriminated based on probe ps8, which forms a perfect duplex only with *B. thuringiensis* 4Q281 (FIG. 4, Table 4).

Therefore, hybridization of 16S rRNA from bacteria of the *B. cereus* group with probes from region 77-92 can be used to discriminate microorganisms of subgroup Thuringiensis B from bacteria of all other subgroups, and it is especially important for the discrimination between subgroups Thuringiensis A and B.

Example 10: Identification of *B. anthracis* Ames (subgroup Anthracis)

Probe ps17 is specific for subgroups Anthracis, Cereus A, and Mycoides B, forming perfect duplexes with 16S rRNA from *B. anthracis* Ames, *B. thuringiensis* B8 and *B. mycoides* 6462m/10206, and mismatches with all other reference microorganisms (FIG.1 and Table 4). In contrast, probe ps18 contains a mismatch for *B. anthracis* Ames, *B. thuringiensis* B8 and *B. mycoides* ATCC 6462/ATCC10206, and is a perfect match with all other references microorganisms. Discrimination of *B. anthracis* Ames and *B. thuringiensis* B8 from *B. mycoides* ATCC 6462/ATCC10206 can be based on a "perfect" signal for probe ps17 (compare with ps18) in combination with "mismatch" signal for probe set #1 (FIG. 4, Table 4). Microorganisms of subgroup Mycoides A have one additional mismatch with probes ps17 and ps18.

Example 11: Identification of *B. cereus* T (subgroup Cereus B)

Identification of *B. cereus* (strain T) can be established based on perfect match signals for probes ps18, ps7(ps9), and for probe sets #2 and #4 (FIG.1, 4 Table 4).

Example 12: Identification of *B. thuringiensis* B8 (subgroup Cereus A)

Organisms that belong to subgroup Cereus A contain 16S rRNA sequences that are identical to *B. anthracis* Ames (subgroup Anthracis) or that differ from *B. anthracis* Ames by strain-specific sequence variation only (Table 2). Thus, 23S rRNA sequences were used to differentiate bacteria from subgroup Cereus A and *B. anthracis* Ames. The 23S rRNA sequences of *B. thuringiensis* B8 and *B. cereus* NCTC11143 differ from *B. anthracis* Ames at three sites, Y/C (594), insertion G (1218-1219) and G/A (1559) (FIG. 2, Table 3). Two pairs of probes were used, ps21/ps22 and ps23/ps24 (FIG 2) to target sites 1559 and 1219, respectively. Probes ps21 and ps23 form a perfect duplex with the 23S rRNA of *B. anthracis* Ames but not *B. thuringiensis* B8 and *B. cereus* NCTC11143 23S rRNA. Probes ps22 and ps24

provide complementary information, by having a mismatch with *B. anthracis* Ames 23S rRNA and being complementary to *B. thuringiensis* B8 and *B. cereus* NCTC11143 23S rRNAs (FIGS. 2, 5A). The discriminative feasibility of 23S rRNA probes ps21/ps22 and ps23/ps24 was tested by using *B. cereus* HER1414, whose rRNA genes were not sequenced. *B. cereus* HER1414 and *B. anthracis* Sterne revealed the same hybridization pattern after hybridization with a 16S rRNA microchip (FIG. 5B, ps18/ps17 shown only). A set of 23S rRNA probe pairs ps21/ps22 and ps23/ps24 successfully discriminated *B. cereus* HER1414 and *B. thuringiensis* B8 from *B. anthracis* Sterne with the hybridization signal ratio for these two pairs being 0.7, 0.4, 2.2 and 0.7, 0.4, 1.7, respectively (FIG. 5B).

Example 13: Identification of polymorphic sites

Partial (about 80% of total) sequencing of the *B. anthracis* Ames genome (TIGR website http://www.tigr.org) indicated that organisms from the *B. cereus* group may contain at least ten copies of the rRNA operon. Considerable polymorphism of these genes at some sites (for example 1:1 ratio of C:T at position 594 of 23S rRNA) has been demonstrated (TIGR website http://www.tigr.org). Sequencing revealed two polymorphic sites in the 23S rRNA molecule at positions 594 and 1559 for a number of microorganisms of the *B. cereus* group (FIG. 2 and Table 3). For example *B. cereus* T, *B. thuringiensis* 4Q281 and *B. cereus* 9620 have polymorphisms in their 23S rRNA genes at site 1559 with G:A ratios equal to 1.5:1, 1:1 and 1:3.5, respectively. At the same time, *B. anthracis* Ames and *B. mycoides* 10206 have a G in this site, and *B. thuringiensis* B8 has an A in this position (FIG. 2). The possibility of recognizing polymorphisms in this site was demonstrated by using a pair of probes, ps21/ps22. For these probes the hybridization signal ratio for *B. cereus* T, *B. thuringiensis* 4Q281 and *B. cereus* 9620 have been found to be intermediate (1.0, 0.72 and 0.64, respectively) between *B. anthracis* Ames, or *B. mycoides* 10206 (1.8 and 1.5, respectively) and *B. thuringiensis* B8 (0.45) (FIG. 5A).

Example 14: Identification of mixtures

The 16S rRNA of *B. cereus* 9620 and *B. anthracis* Ames differ from each other in only one nucleotide. Nevertheless, not only homogeneous samples of these bacteria were discriminated, but also mixtures of RNA from these two microorganisms (FIG. 6A). The ratio of probes ps17/ps18, species-specific to these

bacteria, is 1:3 for *B. cereus* 9620 and 8:1 for *B. anthracis* Ames. A 2:3 mixture of RNA from *B. cereus* 9620 and *B. anthracis* Ames, respectively, revealed 3:2 ratio (FIG. 6A). Identification of 10:1 mixture of *B. thuringiensis* B8 RNA and *B. thuringiensis* 4Q281 RNA were demonstrated. The identification of microorganisms and their relative content in the mixture was determined using three pairs, ps5/ps6, ps7/ps8, and ps17/ps18 (FIG 6B). The signal changes were different for different probe pairs. For correct estimation of the percentage in the mixture, a calibration would be required for each pair separately. Even without quantitative interpretation of the results variance from the expected hybridization ratios suggests the presence of both target groups.

Example 15: Cross-hybridization of selected probes with non-target bacteria

The number of subgroup-specific differences in rRNA sequences for bacteria from the *B. cereus* group are few and localized. This creates difficulties in the selection of probes specific to individual subgroups of bacteria. For example, the sequences of two of the twenty probes on the microchip (ps17 and ps20) (FIG. 1) selected for identification of *B. cereus* group reference microorganisms also match the 16S rRNA sequences of a number of bacteria that belong to other groups of the genus *Bacillus*. The use of probes targeting the entire *B. cereus* group and for some other groups may resolve this problem. To demonstrate this, probe ps25 was designed to match all known sequences of *B. cereus* microorganisms and probe ps26 to target the *B. subtilis group* (*B. subtilis, B. amiloliquifaciens, B. lentimorbus, B. popilliae,* and *B. atrophaeus*). Hybridization analysis indicated that these probes effectively differentiated *B. subtilis* B-459 from *B. anthracis* Ames, *B. cereus* T, *B. mycoides* 10206, and *B. thuringiensis* 4Q281 (FIG.7). Thus, the *B. cereus* group probes can be used as an internal check to validate probes ps17 and ps20.

MATERIALS AND METHODS

Bacterial strains

Ten strains belonging to the *B. cereus* group: *B. anthracis* str. Ames ANR, *B. anthracis* str. DeltaAmes-1, *B. thuringiensis* str. B8, *B. cereus* str. NCTC9620, *B. cereus* str. T, *B. thuringiensis* str. 4Q281, *B. medusa* str. ATCC25621, *B. mycoides* str ATCC6462m, and *B. mycoides* str. ATCC10206 (Table 2) were obtained from Dr. John Ezzell, USAMRIID (The U.S. Army Medical Research Institute of Infectious

Diseases, Fort Detrick, Maryland). Two of these strains were isolated as an occasional admixture from a culture previously identified as *B. mycoides* str. ATCC6462, revealed different colony morphology and received strain numbers, *B. mycoides* str. ATCC6462m and *B. mycoides* str. ATCC10206.

Sequencing of 16S and 23S rRNA genes

Total DNA was isolated from frozen cell pellets by the guanidine extraction method. 16S rDNA and 23S rRNA were amplified from total genomic DNA for 10 strains. For each amplification reaction, 0.1 μg of bacterial DNA was subjected to PCR in a total volume of 100 μl, with 2.5 units of Taq polymerase (Perkin-Elmer, Boston, MA), 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 200 μM of each dNTP (dATP, dCTP, dGTP, TTP), and 6 μM of each of two primers. The primers used for 16S rDNA and 23S rDNA amplification are listed in Table 1. The thermal profile included denaturation at 94°C for 2 min, primer annealing at 45°C for 2 min, and extension at 72°C for 2 min and then 35 cycles of denaturation at 94°C for 15 sec, primer annealing at 45°C for 15 sec, and extension at 72°C for 4 min. DNA was purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and purified PCR products were directly sequenced by the cycle sequencing method using AmpliTaq DNA polymerase FS (Perkin-Elmer, Boston, MA), fluorescently labeled dye-terminators, and 373A fluorescent sequencer (ABI; Perkin-Elmer, Boston, MA). Sequencing primers are shown in Table 1.

Development of Expanded Sequence Databases

All 16S and 23S rRNA sequences for members of the *B. cereus* group available in GenBank were retrieved. Thirty-three 16S rRNA sequences obtained from the GenBank database and one from "The Institute of Genomic Research (TIGR) were aligned with eleven 16S rRNA sequences independently determined herein, including one *B. anthracis* strain (Sterne) resequenced is part of the invention (Table 2). Software developed in the inventors' lab was used for comparative analysis and probe design.

Six complete and five partial sequences of 23S rRNA for *B. cereus* group bacteria were obtained from GenBank and TIGR. These were aligned with nine additional 23S rRNA sequences for the *B. cereus* group determined in this study and one *B. anthracis* strain (Sterne) resequenced as part of the invention (Table 3).

Creation of Phylogenetic Tree

The 16S and 23S rRNA sequence databases were also used to create an unrooted phylogenetic tree for all of the strains in the database. These trees were created using the multiple sequence alignment computer program "Clustalx". All positions of nucleotides in analyzed alignment of sequences, where unidentified nucleotides N were found, were excluded from consideration for all microorganisms whose sequences were included in the alignment.

Design of oligonucleotide probes

The following strategy was used for the probe design. Each unique 16S rRNA sequence was used to create a set of all 20-mer oligonucleotides possible for that sequence (the set consisted of L-19 overlapping oligonucleotides, where L denotes the length of the entire 16S rRNA sequence). Each of these 20-mer overlapping oligonucleotides was then considered as a potential probe. Each potential probe was tested against all available 16S rRNA sequences (GenBank and RDP) by an algorithm that estimates the relative duplex stability according to the number and position of mismatches. If the 16S rRNA of any microorganism that did not belong to the target group (genus, species, or subgroup) of interest formed stable duplexes with any oligonucleotide considered as a potential probe for the microchip, this oligonucleotide was excluded from the list of probes, except probes ps17 and ps20 specifically mentioned in Example 15.

Oligonucleotide microchip design and construction

DNA microchips were constructed with ten or 31 pairs of oligonucleotide probes targeting 16S rRNA and 23S rRNA sequences (see FIG. 1 and FIG. 8) and two group-specific probes (ps25 and ps26), two pairs of oligonucleotide probes targeting 23S rRNA sequences (ps21 and ps22, see FIG 2) except probe ps26 which was 19 bases long. Each probe was 20 bases long. Oligonucleotides were synthesized on an automatic DNA/RNA synthesizer (Applied Biosystems 394) using standard phosphoramide chemistry. 5'-Amino-Modifier C6 (Glen Research, Sterling, VA) was linked to the 5'-end of the oligonucleotides. A micromatrix containing 100- by 100- by 20 μm polyacrylamide gel pads fixed on a glass slide and spaced 200 μm from each other was manufactured by photopolymerization, and activated as described herein. Six nl of individual 1mM amino-oligonucleotide solutions was applied to each

gel pad containing aldehyde groups according to the procedure described below.

Determination of Relative Duplex Stability

To determine the realtive duplex stability wherein the amount of positions where the microorganisms may be differentiated was restricted, and were tested all positions synthesizing all reasonable oligo pairs around each site of differentitation, oligos were applied onto the chip and hybridized with labeled RNA from appropriate microorganisms. Pairs of oligos that revealed the highest signal in combination with highest perfect signal/mismatch signal ratio were selected.

Preparation of Acrylamide Micro-Matrices by Photo-Polymerization

Preparation of glass slides

1. Immerse 10 glass slides in 10 M sodium hydroxide in a Wheaton glass-slide container (volume 150 ml) for 30 minutes.
2. Rinse with five changes of double-distilled water in a container.
3. Immerse 10 slides in concentrated sulfuric acid in container for 30 minutes
4. Rinse five times in double-distilled water and allow to stand in double distilled water for 5 minutes then rinse again.
5. Remove water drops with nitrogen stream. Dry for 1 h at 60oC.

Treatment of cleaned slide with Bind Silane.

Immerse slides in 3-(Trimethoxysilyl)propyl methacrylate and incubate for 40 h min at 37°C.

Rinse thoroughly with ethanol and then double-distilled water and dry under a nitrogen stream.

Preparation of solutions for aldehyde matrices

1. Composition of 5% polyacrylamide solution

0.5 ml 40% Acrylamide /Bis solution (19:1)

1.82 ml 0.2M sodium phosphate buffer (consists of equal volumes of 0.2M sodium phosphate monobasic monohydrate and 0.2M sodium phosphate dibasic

anhydrous pH =6.8, store at 4°C).

1.6 ml glycerol

0.08 ml monomer I solution (N-(5,6-di-O-isopropylidene)hexyl acrylamide). For monomer I solution add 1 ml MilliQ water to aliquot of monomer I stock (25 mg) located in –80oC freezer. Aliquot and store these at –20oC for approximately 1 month.

2. filter.

3. Prepare solution weekly and store at 4°C. Allow solution to reach room temperature before use.

Assembly of gel-casting cassette.

1. Clean mask surface with ethanol.
2. Rinse thoroughly with distilled water stream rubbing briskly with lint-free tissue.
3. Dry under a nitrogen stream.
4. Place spacers (audio tape film) on chrome side of mask; two spacers from both sides and one in the center.
5. Place slide over mask and spacers with treated surface facing mask.
6. Clamp in place.

Photo-polymerization (optimized for 4-cluster mask).

Prepare mixture: 100µl of above polyacrylamide solution
0.4 µl Methylene blue (0.04%)
1.2µl TEMED
Vortex 3 seconds
Degas solution 3 min

1. Pipette mixture between the slide and the mask allowing the solution to move between the space by capillary action. Take care that air does not enter the pipette or space. Pipette off excess solution.

2. Turn cassette over so that glass slide is underneath the mask. Place in Oriel chamber.
3. Irradiate for 300 sec.
4. Carefully disassemble the cassette under water using the point of a scalpel to separate the slide and mask (the slide floats free without pressure being placed on the gel elements.) Take care not to scratch mask.
5. Rinse 30 seconds under running distilled water and soak in distilled water for 15 minutes
6. Air dry in a laminar-flow hood
7. Keep in dust -free slide box at room temperature. Matrices can be kept for at least 1 year.

Procedure for activation (deprotection) of aldehyde matrices

1. Place matrix in 2% trifluoro-acetic acid for 10 minutes at room temperature (prepare fresh solution after every 10 microchips).
2. Rinse well (5 or 6 times) with filtered distilled water for 1 min
3. Wash in distilled water X3 times then leave 3-5 mins in last rinse and dry 20 min in air.
4. Put slide into Repel Silane™ (use fresh solution for each treatment) for one minute.
5. Wash with acetone or dichloromethane (15 sec) and then thoroughly with tap - distilled water (15 sec under stream).
6. Load oligonucleotides.
7. Put microchip into freshly prepared solution of pyridine-borane complex in chloroform (0.1M)(80 ml chloroform/1ml pyridine borane) and cover chloroform layer with water; (approx ¼ inch)hold 12 hours at room temperature (O. N.)
8. Wash microchip with distilled water.
9. Place microchip into 0.1M sodium borohydride on microchip for 20 min .
10. Wash with distilled water 1 min.

11. Heat microchip in 0.1X SSPE with 0.1% SDS at 60°C for 1h (50 ml).
12. Wash biochip in Hybridization Station for 15 min on a stirrer.
13. Wash with distilled water 1 min.
14. Dry microchip in a dust-free environment in the air for 20 min.
15. The chip is now ready for hybridization. The chip could be kept at room temperature.

Standardized Sources of Chemicals and Equipment

1. DEPC-Treated Water (Ambion, cat#9920)
2. 0.5M EDTA, pH 8.0 (Ambion, cat#9260G)
3. Eppendorf Centrifuge 5417C (Fisher, cat#05-406-11)
4. Eppendorf microcentrifuge tubes, 1.5ml (Fisher, cat#05-402-24B)
5. Acetone (Sigma, cat#A4206)
6. Guanidine Thiocyanate (Fisher, cat#BP221-1)
7. 1M HEPES (Sigma, cat#H4034)
8. Hybridization chamber: Probe-Clip Press-Seal Incubation Chamber (Sigma, cat#Z36,157-7)
9. Kimwipes (Fisher, cat#06-666A)
10. 20X SSPE (Sigma, cat#S2015)
11. Tween 20 (Fisher, cat#BP337-100)
12. Imaging Chamber (Sigma, cat#Z36,585-8)
13. Ultrafree-MC 0.45 μm filter unit (Millipore, cat#UFC30HVNB)
14. Triton X-100 (Sigma, cat#T9284)
15. Ethyl Alcohol, absolute 200 proof, 99.5%, A.C.S. reagent (Aldrich, cat#45,984-4)
16. QIAquick PCR Purification Kit (50) (Qiagen, cat#28104)
17. Taq DNA Polymerase (includes 10× PCR reaction buffer) (Amersham Pharmacia Biotech, cat#T0303Z)

18. PCR Nucleotide Mix: PCR nucleotide mix (10 mM each dATP, dCTP, dGTP, dTTP) (Amersham Pharmacia Biotech, cat# US77212)
19. Sonicated Salmon Sperm DNA, Phenol Extracted (Amersham Pharmacia Biotech, cat#27-4565-01)
20. Albumin from bovine serum, 20 mg/ml in water (Sigma, cat#B8667)
21. Luer Lok syringe, 60 cc/2oz, B-D (Fisher cat#14-823-2D)
22. Millex-GN 0.20 filter units (Millipore, cat#SLGN025NS)

An example of a customized microchip is shown in FIG. 8 and Table 5.

RNA isolation

Total RNA was isolated from frozen cell pellets of eight *B. cereus* group bacteria: *B. anthracis* Ames, *B. thuringiensis* B8, *B. cereus* T, *B. cereus* 9620, *B. thuringiensis* 4Q281, *B. medusa* 25621, *B. mycoides* 6462m and *B. mycoides* 10206. Cells were lysed by bead beating the cell pellet (approx. 0.2 g) in the presence of 0.5 g baked zirconium oxide beads (0.1 mm), 500 µl phenol (pH 5.1), 500 µl 0.1 M sodium acetate, pH 5.1, and 50 µl 20% SDS at high speed for 2 min, followed by incubation at 65°C for 10 minutes and an additional bead-beating step for 2 minutes at high speed. RNA was isolated by phenol extraction and precipitated by addition of 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol. Following storage at -80°C overnight the RNA was recovered by centrifugation at 14,000 rpm for 5 min, washing the RNA pellets two times with ethanol, and resuspending in diethyl pyrocarbonate (DEPC)-treated H_2O.

Magnesium-sodium periodate fragmentation of RNA and dye labeling

RNA (10 to 20 µg) and DEPC treated H_20 were combined and preheated at 95°C for 5 min. $MgCl_2$ was added to 60 mM (total volume 20 µl) and the reaction solution was heated at 95°C for 40 min. Phosphatase treatment was carried out by addition of 3 µl 10X alkaline phosphatase buffer (Promega, Madison, WI) and 0.2 µl alkaline phosphatase (1 U/µl) (Promega, Madison, WI) and heating at 37°C for 30 min. Oxidation of the 3'-end ribose moiety was conducted by addition of 6.5 µl of 100 mM sodium periodate and incubation at room temperature for 20 min. Labeling was carried out by addition of 3.5 µl of 100 mM Lissamine rhodamine B

ethylenediamine (LissRhod) (Molecular Probes, Eugene, OR), 1.65 µl of 1 M HEPES (pH 7.5) and heating at 37°C for 1 h. Reduction of Schiff base was conducted by addition of 6.7 µl of 200 mM sodium cyanoborohydride and incubation at room temperature for 30 min. Labeled RNA was precipitated by addition of 15 volumes of 2% lithium perchlorate in acetone and storage at -20°C for 20 min. After centrifugation at 14,000 rpm for 5 min, RNA pellets were washed twice with acetone and dried at 55°C for 10 min.

Excess LissRhod was removed from RNA by butanol treatment: RNA pellets were suspended in 300 µl DEPC-treated H2O, and samples were concentrated to 60 µl by removal of water with butanol. Treatment was repeated until butanol was free of color. RNA was precipitated in 2% LiClO4 in acetone at -20°C for 20 min. After centrifugation at 14,000 rpm for 5 min, RNA pellets were washed twice with acetone, dried at 55°C for 10 min, and suspended in 10 to 20 µl DEPC treated H2O.

Hybridization with microchips

The 35 µl of hybridization solution containing 0.1 to 1 µg fragmented and labeled RNA, 1M guanidine thiocyanate (GuSCN), 5mM EDTA, and 40mM HEPES (pH 7.5) was passed through a 0.22 µ filter to remove particulates, then heated at 95°C for 3 min and placed on ice. Thirty µl of the hybridization solution was added to a hybridization chamber (Grace Biolabs, Bend, OR), and the hybridization chamber was affixed to a microchip. The microchip was allowed to hybridize overnight at room temperature in the dark. After hybridization, the chamber and hybridization solution were removed from the microchip, and the microchip was washed twice for 10 sec each with 100 µl washing buffer. Washing buffer consisted of 0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 6 mM EDTA, and 1% Tween 20.

Hybridization data analysis

After hybridization the microchips were analyzed with a custom made wide-field-high-aperture fluorescence microscope (Vavilov State Optical Institute, St. Petersburg, Russia) equipped with a cooled CCD camera (Princeton Instruments, Acton, MA), a thermal table, and XY positioners, and operated with a computer with specially designed software. Parameters of the microscope are as follows: field of view 4 mm by 4 mm, aperture 0.4, working distance 12 mm. Up to 144 individual gel elements with the size of 100- by 100- by 20 µm spaced by 100 µm may be analyzed

in parallel in one field of view. Images were captured with WinView Software (Princeton Instruments). The hybridization data was quantified from the WinView image using software (Lab View, version 4.0.1 and MicroChip Imager, Oleg Alferov).

Table 1. Primers used for PCR and for sequencing of 16S and 23S rRNA genes of *B. cereus* groups bacteria [(a)].

Name	Sequence	Location
P1	5' - GTT TGA TCC TGG CTC AG	11 - 27 (16S rRNA)
P10	5' - CCA GTC TTA TGG GCA GGT TAC	136 –116 (16S rRNA)
P11	5' - TCC ATA AGT GAC AGC CGA AGC	226 - 206 (16S rRNA)
P5	5' - CTA CGG GAG GCA GCA GTG GG	340 - 360 (16S rRNA)
P3	5' - GWA TTA CCG CGG CKG CTG	535 -517 (16S rRNA)
P2	5' - GGA TTA GAT ACC CTG GTA GT	784 - 803 (16S rRNA)
P6	5' - CCG TCA ATT CCT TTR AGT TT	926 - 907 (16S rRNA)
P8	5' - TTC GGG AGC AGA GTG ACA GGT	1029 - 1049 (16S rRNA)
P9	5' - TAC ACA CCG CCC GTC ACA CCA	1392 - 1412 (16S rRNA)
P4	5' - RGT GAG CTR TTA CGC	1513 - 1492 (16S rRNA)
Pr1	5' - CCG AAT GGG GVA ACC C	114 - 129 (23S rRNA)
Pr13	5' - CCG TTT CGC TCG CCG CTA CTC	262 - 242 (23S rRNA)
PB1	5' - TAG TGA TCG ATA GTG AAC CAG	485 - 505 (23S rRNA)
Pr2	5' - CAT TMT ACA AAA GGY ACG C	621 - 603 (23S rRNA)
Pr3	5' - GCG TRC CTT TTG TAK AAT G	603 - 621 (23S rRNA)
PB2	5' - TAG TGA TCG ATA GTG AAC CAG	755 - 736 (23S rRNA)
PB3	5' - TAG TGA TCG ATA GTG AAC CAG	969 - 990 (23S rRNA)
Pr4	5' - RGT GAG CTR TTA CGC	1151 - 1137 (23S rRNA)
Pr5	5' - WGC GTA AYA GCT CAC	1136 - 1150 (23S rRNA)
PB4	5' - CAT ACC GGC ATT CTC ACT TC	1308 - 1289 (23S rRNA)
PB5	5' - ACA GGC GTA GGC GAT GGA C	1408 - 1426 (23S rRNA)
PB8	5' - AAC CTT TGG GCG CCT CC	1679 - 1661 (23S rRNA)
Pr6	5' - CYA CCT GTG WCG GTT T	1673 - 1659 (23S rRNA)
Pr7	5' - AAA CCG WCA CAG GTR G	1659 - 1673 (23S rRNA)
Pr8	5' - CAY GGG GTC TTT RCG TC	2092 - 2076 (23S rRNA)
Pr9	5' - GAC GYA AAG ACC CCR TG	2076 - 2092 (23S rRNA)
Pr10	5' - GAG YCG ACA TCG AGG	2535 - 2521 (23S rRNA)
Pr11	5' - CCT CGA TGT CGR CTC	2521 - 2535 (23S rRNA)
Pr12	5' - GYT TAG ATG CYT TC	2783 - 2770 (23S rRNA)
R1	5' - GGC GGC GTC CTA CTC TCA C	112 - 95 (5S rRNA)

[(a)] - primers P1-P4, Pr1-Pr7 and R1 were used for DNA amplification. All other primers were utilized for sequencing. Primers P8, P9, P10 and P11 were selected *de novo*, other primers were described previously.

Table 2. Classification of bacteria in the *Bacillus cereus* group according to 16S rRNA sequences

Subgroup name	Subgroup-specific signatures (position) [*]	Start and end of sequence	Organism	GenBank AC	Positions of strain-specific variations
Anthracis	Consensus	11 – 1556	***B.anthracis str. Ames ANR*** [a,e]	AF155950, TIGR [g]	-
		11 – 1556	*B.anthracis str. DeltaAmes-1* [a]	AF155951	-
		11 – 1556	*B.anthracis str. Sterne* [a]	AF176321 [a]	-
		1 – 1453	*B.anthracis str. Sterne*	X55059	del C(942)[b]
		61 - 528, 815 - 1503	*B.anthracis str. 1*	***	-
		61 - 528, 815 - 1503	*B.anthracis str. 2*	***	-
Cereus A	Consensus	11 – 1556	***B.thuringiensis str. B8*** [a,e]	AF155955	-
		1 – 1453	*B.cereus str. NCTC11143*	X55063	-
		-	*B. cereus str. HER1414* [f]	-	?
		31 – 1464	*B.sp. str. JJ#1* [h]	Y15466	-
		28 – 1536	*B.sp. str. BSID723* [h]	AF027659	1232
		49 – 1524	*B.cereus str. WSBC10037* [h]	Z84576	178
		49 – 1524	*B.cereus str. WSBC10030* [h]	Z84575	353, 600, 864, 1146
		28 – 1515	*B.cereus str. 1396* [h]	AF206326	181, 467, 480, 482, 590, 995, 1146, 1244, 1345
Cereus B	***C/A(1015)***	11 – 1556	***B.cereus str. NCTC9620*** [a,e]	AF155952	-
		11 – 1556	***B.cereus str. T*** [a,e]	AF176322	-
		28 – 1515	*B.cereus str. IAM12605* [i]	D16266	-
		1 – 1453	*B.cereus str.NCDO1771* [i]	X55060	-
		49 – 1524	*B.cereus str. ATCC27877*	Z84581	828
		26 – 1183	*B.cereus*	AF076031	498, 520, 523, 829, 1167
		49 – 1524	*B.thuringiensis str.WS2614*	Z84584	127
		49 – 1524	*B.thuringiensis str. WS2617*	Z84585	1153
		49 – 1524	*B.thuringiensis str. WS2618*	Z84586	725
		49 – 1524	*B.thuringiensis str. WS2626*	Z84588	-
Thuringiensis A	***C/A(1015)***	49 – 1524	*B.thuringiensis str. WS2623*	Y18473	109, 679, 1228, 1505
		49 – 1524	*B.thuringiensis str. WS2625*	Z84587	565, 1183
	C/T(192)				
Thuringiensis B	***C/A(1015)***	11 – 1556	***B.thuringiensis str. 4Q281*** [a,e]	AF155954	-
		11 – 1556	***B.medusa str ATCC25621*** [a,c,e]	AF155958	no C/T (192)
	C/T(192)	11 – 1556	*B.medusa.str. NCIMB10437* [c]	X60628	1038, 1383
		28 – 1515	*B.thuringiensis str. IAM12077* [j]	D16281	-
	A/G(77), T/C(90),	1 – 1453	*B.thuringiensis str. NCIMB9134* [j]	X55062	-
	T/A(92)	7 – 1552	*B.sp. str. Termite isol. 'bac'*	X81132	182,186,187,769,822,823,1281,1282,1283,1301,1429,1430, 1463,1464,1475, 1476,1478,1479
Mycoides A	***C/A(1015)***	1 – 1453	*B.mycoides str. DSM2048* [d]	X55061	-
		49 – 1556	*B.mycoides str. MWS5303-1-4*	Z84591	1456
	C/T(192)	49 – 1526	*B.mycoides str. DRC1*	AF144645	63, 1279, 1319-1321, 1398, 1439, 1443, 1473, 1479, 1484
		49 – 1524	*B.mycoides str. MWS5303-2-51*	Z84583	180
	G/A(133), C/T(182),	32 – 1546	*B.mycoides str. ATCC6462* [d]	AB021192	-

	G/A(197), A/G(286) C/T(1019), G/A(1030), T/A(1462)	14 – 1546 49 – 1524 49 – 1524 49 – 1524 49 – 1524	*B.weihenstephanensis DSM11821* *B.cereus str. WSBC10201* *B.cereus str. WSBC10204* *B.cereus str. WSBC10206* *B.cereus str. WSBC10210*	AB021199 Z84577 Z84578 Z84579 Z84580	- 203, no A/G(286), 1515 128 225, 1519, 1520 60, 375, 1298
Mycoides B	A/C(189), T/G(200), G/C(208), T/C(1036), A/G(1045)	11 – 1556 11 – 1556 34 – 1374 7 – 1538	***B.mycoides str ATCC6462m*** [a,e] ***B.mycoides str ATCC10206*** [a,e] *B.cereus str. Ki21* *B.pseudomycoides sp. nov.*	AF155956 AF155957 AJ288157 AF013121	- - 95, no T/G(200), 202, 329, 752, 778, 793, no T/C(1036), 1350, 1360, 1374 55, 341, 495, 516, 566, 929, 1017, 1104, 1110, 1121, 1128, 1138

(*) - for more details see Fig. 1. [a] - sequenced in this work. [b] - need to be reexamined, see also Results. [c] - according to Bergey's Manual, these two strains of *B. medusa* should be identical. This was not confirmed with 16S rRNA sequencing. [d] - according to Bergey's Manual, these two strains of *B. mycoides* should be identical. [e] - strains selected in this study as reference organisms to demonstrate subgroup identifications. [f] - not sequenced, identified in this study by 16S/23S rRNA oligonucleotide microchip analysis. [g] - partial (about 90%) sequences of whole *B. anthracis str. Ames* genome, data of TIGR (http://www.tigr.org). [h] - final discrimination from Anthracis subgroup will be done after testing 23S rRNA gene sequence. *** - J. Jackman, unpublished. [i] - synonym of *B. cereus str. DSM31* (see also Table 3). [j] – synonym of *B. thuringiensis str. DSM 2046* (see also Table 3).
Subgroup-specific mutations, which are highlighted in bold, are identical for two or more subgroups and were placed on separate lines to demonstrate connections between different subgroups.

Table 3. Classification of bacteria in the *Bacillus cereus* group according to 23S rRNA sequences

Subgroup name	Subgroup-specific signatures (position) [*]	Start and end of sequence	Organism	GenBank AC	Positions of strain-specific variations
Anthracis	Consensus	1 – 2922	***B.anthracis str. Ames ANR*** [a,d]	AF267734 [a], TIGR [b]	-
		1 – 2922	*B.anthracis str. DeltaAmes-1* [a]	AF267876	-
		1 – 2922	*B.anthracis str. Sterne* [a]	AF267877 [a]	-
		15 – 2943	*B.anthracis str. Sterne*	S43426	T/C(491) [e], del CG(1413, 1414) [e], T/C(2651) [e]
Cereus A	*Y/C(594)*	1 – 2923	***B.thuringiensis str. B8*** [a,d]	AF267880	-
		1 - 2923	*B.cereus str. NCTC11143*	X64646	-
	G/A(1559)	1 – 527	*B.cereus str. WSBC10030* [f]	Z84589	-
		-	*B.cereus str. HER1414* [c]	-	?
	Insertion G(1218-1219)				
Cereus B	*Y/C(594)*	1 – 2922	***B.cereus str. NCTC9620*** [a,d]	AF267878	-
		1 – 2922	***B.cereus str. T*** [a,d]	AF267879	-
	G/R(1559)	1 - 2787	*B.cereus str. DSM31* [g]	X94448	T/C(1275), G/A(1559)
		1 - 527	*B.thuringiensis str. WS2617* [f]	Z84594	-
	T/A(2153)	1 - 527	*B.thuringiensis str WS2614* [f]	Z84593	-
Thuringiensis B	Y/T(594)	1 – 2922	***B.thuringiensis str. 4Q281*** [a,d]	AF267881	G/R(1559)
		1 – 2922	***B.medusa str. ATCC25621*** [a,d]	AF267885	-
	T/C(157)	1 – 2784	*B.thuringiensis str. DSM2046* [h]	X89895	C/T(57), T/G(413), ins(AATA)(479-480), del(GG)(541-542), G/A(646), C/G(670), G/A(1953), G/A(2055), ins(AGT)(2556-2557), del(G)(2573)
	G/A(921), A/G(1020), C/T(1037), G/A(1209), A/G(1251), T/C(1283) C/T(132), A/T(174) T/A(2153) G/T(1250)				
Mycoides A	*T/C(157)*	1 - 527	*B.mycoides str. 2048T*	Z84592Z84591	-
		1 - 527	*B.mycoides str. MWS5303-1-4*	Z84590	-
	CA/TC(265,266), GT/AC(364,365)		*B.cereus str. WSBC10206*		-
	C/T(132), A/T(174)				
	C/T(375)				
Mycoides B	Y/T(594)	1 – 2922	***B.mycoides str. ATCC6462m*** [a,d]	AF267884	-
		1 – 2922	***B.mycoides str. ATCC10206*** [a,d]	AF267883	-
	T/C(157)				
	G/A(921), A/G(1020), C/T(1037), G/A(1209), A/G(1251), T/C(1283) CA/TC(265,266), GT/AC(364,365)				
	GA/AG(346,347), TC/CT(358,359), C/A(482), C/T(672), A/T(1219), G/T(1268),				

	C/G(1816), G/C(1849), A/G(2159)				

(*) - for more details see Fig. 2. [a] - 23S rDNA sequenced in this work. [b] - partial (about 90%) sequences of whole *B.anthracis str. Ames* genome, data of TIGR (http://www.tigr.org). [c] - not sequenced, identified in this study by 16S/23S rRNA oligonucleotide microchip analysis.
[d] - strains selected in this study as reference organisms to demonstrate subgroup identifications. [e] - need to be reexamined. [f] - final subgroup discrimination will be done after completion of 23S rRNA sequencing. [g] - synonym of *B.cereus str. IAM 12605* and *B.cereus str. NCDO1771* (see Table 2). [h] –synomym of *B.thuringensis str. IAM12077* and *B.thuringiensis str. NCIMB 9134* (see Table 2). R = G, or A. Y = T, or C.
Subgroup-specific mutations, which are highlighted in bold, italics or underline denote mutations that are identical for two or more subgroups, and were grouped to demonstrate connections between different subgroups

Table 4. Degree of matching between oligonucleotide probes contained on microchip and the 16S and 23S rRNA sequences of eight reference microorganisms from the *B.cereus* group(*)

Probes	Probe's target	***B. thur* + *B. med*** / **all other**			***B. thur*** / all other	***B. mycoides*** / **all other**					***B. anthr.* + *B. myc*** / **all other**	all other / ***B. thur. B8***
	Probe's name	ps1 / ps2	ps3 / ps4	ps5 / ps6	ps7 / ps8	ps9 / ps10	ps11 / ps12	ps13 / ps14	ps15 / ps16	ps19 / ps20	ps17 / ps18	ps21 / ps22
Reference organisms	*B. anthracis AMES*	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
	B. thuringiensis B8 (*B. anthracis* mimic)	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+
	B. thuringiensis 4Q281	-/+	-/+	-/+	-/+	+/-2	+/-2	+/-	+/-	+/-	-/+	p
	B. cereus T	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+	p
	B. cereus NCTC9620	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+	p
	B. medusa ATCC25621	-/+	-/+	-/+	+/-	+/-	+/-	+/-	+/-	+/-	-/+	p
	B. mycoides ATCC10206	+/-	+/-	+/-	+/-3	-/+	-/+	-/+	-/+	-/+	-/+	+/-
	B. mycoides ATCC6462m	+/-	+/-	+/-	+/-3	-/+	-/+	-/+	-/+	-/+	-/+	+/-

(*) data obtained from sequences of RNA genes for corresponding bacteria and represents the set of predicted signals after hybridization with labeled RNA from reference microorganisms.

\+ denotes perfect matching

\- denotes one mismatch

-2 denotes two mismatches

-3 denotes three mismatches

p denotes polymorphism

B. thur: B. thuringiensis 4Q281

B. med: B. medusa ATCC25621

B. myc: B. mycoides ATCC10206 and *B. mycoides ATCC6462m*

B. anthr: B. anthracis AMES

B. thur. B8: B. thuringiensis B8

Table 5

Title	5'-3' Sequence	Target
23F1	TTT GGG CTA TGT TCC GTT TC	not Mycoides A,B
23F2	TTT GGG CTA gaT TCC GTT TC	Mycoides A,B
23F5	TAC GGG GTT GTT ACC CTC TA	not Mycoides A
23F6	TAC GGG GTT aTT ACC CTC TA	Mycoides A
23F7	CTA CGG GGT TGT TAC CCT CT	not Mycoides A
23F8	CTA CGG GGT TaT TAC CCT CT	Mycoides A
16A1	TCT TAT GGG CAG GTT ACC CA	not Mycoides A
16A2	TCT TAT GGG tAG GTT ACC CA	Mycoides A
16A3	ACG CAT CGT TGC CTT GGT GA	not Mycoides A
16A4	ACG CAT CGT cGC CTT GGT GA	Mycoides A
16A5	CAT CGT TGC CTT GGT GAG CC	not Mycoides A
16A6	CAT CGT cGC CTT GGT GAG CC	Mycoides A
16A7	GCG GCT GGC TCC AAA AAG GT	not Mycoides A
16A8	GCG GCT GGC TCC AtA AAG GT	Mycoides A
16A9	GGC TGG CTC CAA AAA GGT TA	not Mycoides A
16A10	GGC TGG CTC CAt AAA GGT TA	Mycoides A
#54 (ps15)	CGA AGC CGC CTT TCA ATT TC	not Mycoides B
SB25 (ps16)	CGA AGC CGC CTT TgA ATT TC	Mycoides B
SB10 (ps13)	GCC TTT CAA TTT CGA ACC AT	not Mycoides B
SB11 (ps14)	GCC TTT gAA TTT CGc ACC AT	Mycoides B
A7	CCC TCT ACG ACG GAC CTT TC	not Mycoides B
A8	CCC TCT gtG ACG agC CTT TC	Mycoides B
23F3	TTT CCA GGT CGC TTC GTC TA	not Mycoides B
23F4	TTT CCA GGc tGC TTC GTC TA	Mycoides B
SB22 (ps18)	TCT AGG GTT tTC AGA GGA TG	not Anthracis, Cereus A, Mycoides B
SB23 (ps17)	TCT AGG GTT GTC AGA GGA TG	Anthracis, Cereus A, Mycoides B
D1	CCG GTT TCA AAG GCT CCC GC	Anthracis, Cereus A, Mycoides B
D2A	CCG GcT TCA AtG GCT CCC GC	not Anthracis, Cereus A, Mycoides B
B1	GAC CCC TAG TCC AAT CAG TG	Anthracis, Cereus A, B
B2	GAC CCC TAG TtC AAT CAG TG	Thuringiensis B, Mycoides B
B7	GGT ATC AAT CCG CAG CTT CG	Anthracis, Cereus A, B
B8	GGT ATC AAT tCG CAG CTT CG	Thuringiensis B, Mycoides B
C5	ACT TCT AAG CAC TCC ACC AG	Anthracis, Cereus A, B
C6	ACT TCT AAG CgC TCC ACC AG	Thuringiensis B, Mycoides B
C7	TCA CTT CTA AGC ACT CCA CC	Anthracis, Cereus A, B

C8	TCA CTT CTA AGC gCT CCA CC	Thuringiensis B, Mycoides B
A3	ATG TAT TCA GAT AAG GAT AC	Anthracis, Cereus A, B
A4	ATG TAT TCA GgT AAG GAT AC	Thuringiensis B, Mycoides B
23F13	ATA CCA TT- GGT ATC AAT CCG	Anthracis
23F14	TA CCA TTc GGT ATC AAT CCG	Cereus A
23F15	TAC CAT T-G GTA TCA ATC CGC	Anthracis
23F16	AC CAT TcG GTA TCA ATC CGC	Cereus A
B11 (ps23)	CAC TGA TAC CAT T-G GTA TCA	Anthracis
B12 (ps24)	CAC TGA TAC CAT TcG GTA TCA	Cereus A
C9	GCT CAG CCT TCA CGA TAA GC	Anthracis
C10	GCT CAG CCT TtA CGA TAA GC	Cereus A
C11 (ps21)	CAG CTC AGC CTT CAC GAT AA	Anthracis
C12 (ps22)	CAG CTC AGC CTT TAC GAT AA	Cereus A
SB12 (ps7,ps9)	GAA CCA TGC GGT TCA AAA TG	not Thuringiensis A,B
#44 (ps8)	GAA CCA TGC aGT TCA AAA TG	Thuringiensis A, B
SB15 (ps3)	TAA CTT CAT AAG AGC AAG CT	not Thuringiensis B
SB16 (ps4)	TAA CTT CtT gAG AGC AAG CT	Thuringiensis B
SB4 (ps5)	CCG CTA ACT TCA TAA GAG CA	not Thuringiensis B
SB4-1 (ps6)	CCG CTA ACT TCt TgA GAG CA	Thuringiensis B
SB1 (ps1)	AGC TCT TAA TCC ATT CGC TC	not Thuringiensis B
#41 (ps2)	AGC TCT cAA TCC ATT CGC TC	Thuringiensis B
A1	CAT TAC GTA TGG TGG GTT TC	not Thuringiensis B, Mycoides A
A2	CAT TAC GTA TaG TGG GTT TC	Thuringiensis B, Mycoides A
A3	ATG TAT TCA GAT AAG GAT AC	not Thuringiensis B, Mycoides A,B
A4	ATG TAT TCA GgT AAG GAT AC	Thuringiensis B, Mycoides A,B
A5	TCT GTC TTC CTT ACC CTA TG	not Thuringiensis B, Mycoides A
A6	TCT GTC TTC CaT ACC CTA TG	Thuringiensis B, Mycoides A
A9(*)	GCC ATC ACC CgT TAA CGG GC	not Thuringiensis B, Mycoides B
A10(*)	GCC ATC ACC CaT TAA CGG GC	Thuringiensis B, Mycoides B
A11(*)	ACG CCA TCA CCC gTT AAC GG	not Thuringiensis B, Mycoides B
A12(*)	ACG CCA TCA CCC aTT AAC GG	Thuringiensis B, Mycoides B
#55	CAA CTA GCA CTT GTT CTT CC	Bacillus cereus group
#57 (ps25)	CGG TCT TGC AGC TCT TTG TA	Bacillus cereus group
#66	ACA GAT TTG TGG GAT TGG CT	Bacillus subtilis group
#67 (ps26)	ATT CCA GCT TCA CGC AGT C	Bacillus subtilis group
SB17 (ps10)	GcA CCA TGC GGT gCA AAA TG	Mycoides B
SB9 (ps11)	CAA TTT CGA ACC ATG CGG TT	not Mycoides B

SB8 (ps12)	gAA TTT CGc ACC ATG CGG Tg	Mycoides B
SB26 (ps19)	TCT GCT CCC GAA GGA GAA GC	not Mycoides B
SB27(ps20)	TCT GCc CCC GAA GGg GAA GC	Mycoides B
Hybr.Marker	GAT GAT GAT GAT GAT GAT GA	Internal standard for hybridization

(*) - bacteria from subgroup Anthracis produce unpredictable results for these

Table 6. Preparation of Buffers for Preparation of Micro-Matrices

Buffer	Chemical/ Solvent/ Elementary buffer	Amount	Final Concentration	Comments
Wash Buffer	20xSSPE buffer	15 ml	3xSSPE	Filter by using Millex GN 0.20 Filter and Luer Lok Syringe, B-D, 60cc/2oz Note: Discard first 5 ml of Wash Buffer when you start filtration Keep at room temperature
	Tween 20	500 µl	1% (v/v)	
	MQ H2O	34.5 ml	-	
3xHybridization Buffer	6 M GuSCN	50 ml	3 M	Store at room temperature
	1 M HEPES, pH 7.5	15 ml	0.15 M	
	0.2 M EDTA, pH 8.0	7.5 ml	15 mM	
	MQ H2O	27.5 ml	-	
Stripping buffer	Guanidinium thiocyanate	300 g	4.9 M	Store solution at room temperature in a bottle with dark glass. Use for 20 stripping procedures (see below) then prepare a new portion.
	1 M HEPES, pH 7.5	13.2 ml	25 mM	
	10% (w/v) Triton X-100	5.2 ml	0.1%	
	Distilled water	250 ml		

NOTE: KEEP ALL BUFFERS IN BOTTLES WITH PLASTIC CAPS

Table 7: Materials and Equipment for Perparation of Micro-Matrices

Chemicals/Equipment	Manufacturer	Catalog #	Lot#
Acetone	Fisher	A18-4	11685
Acrylamide /Bis (19/1) solution 40%	BioRad	161-0144	66767
3-(Trimethoxysilyl)propyl methacrylate	Aldrich	Z-6030	03915TI
Pyridine-borane complex	Aldrich	17,975-2	13905MU
Glycerol	Sigma	G-7893	118H0280
Methylene blue	Merck	73881	51076
Ethyl Alcohol (absolute,200 proof	Aapec Alcohol and Chemical Co	N/A	099I15UA
Chloroform	Aldrich	31,998-8	CO 09980AO
Repel Silane	Amersham-Pharmacia-Biotech	39422	17-332-01
Sodium borohydride	Aldrich	21,346-2	DU 00220MS
Sodium Hydroxide Solid	Sigma	S-5881	11K0116
Sodium Periodate (meta)	Aldrich	S-1878	11K3644
Sodium Phosphate, dibasic. anhydrous	Sigma	S-9763	119H0196
Sodium Phosphate, monobasic monohydrate	Fisher	S-369	792237
Sulfuric Acid	Fisher	A300-500	994173
Sodium Dodecyl Sulfate	Sigma	L3771	83H08411
SSPE, 20X	Sigma	S-2015	107H8508
(N-(5,6-di-O-isopropylidene)hexyl acrylamide)	Argonne, custom made		
TEMED	Sigma	T-7024	67H0136
Trifluoro-acetic Acid	Aldrich	T6,220-0	8K3483
Filter (0.45 μm filter unit); Millex-HV 0.4	Millipore	SLHV 025 LS	

Chemicals/Equipment	**Manufacturer**	**Catalog #**	**Lot#**
Glass slides, 3^2x1^2 Plain;	Corning	2947	
Mask	Nanofilm, California		
Audio tape film	Radioshak XR 60; Type I		
Clamp. Medium Binger clips	Masterbrand	BTM00252	
Oriel Light Source	Oriel Instruments	92532-1000	S/N 139

DOCUMENTS

The following documents are incorporated by reference to the extent they enable the present invention:

Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143-169.

Andersen, G. L., J. M. Simchock, and K. H. Wilson. 1996. Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. 178:377-384.

Aronson, A. I. 1993. Insecticidal toxins, p. 953-963. In A. B. Sonenshein, J. A. Hoch, and R. Losick (ed), Bacillus subtilis and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C

Aronson, A. I., W. Beckman, and P. Dunn. 1986. *Bacillus thuringiensis* and related insect pathogens. Microbiol. Rev. 50:1-24.

Ash, C., and M. D. Collins. 1992. Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-directsequencing. FEMS Microbiol. Lett. 94:75-80.

Ash, C., J. A. E. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins. 1991. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. 41:343-346.

Ash, C., J. A. E. Farrow, W. Wallbanks, and M. D. Collins. 1991. Phylogenetic heterogeneity of the genus Bacillus revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Lett. Appl. Microbiol. 13:202-206.

Barsky, I., A. Grammatin, A. Ivanov, E. Kreindlin, E. Kotova, V. Barsky, and A. D. Mirzabekov. 1998. Luminescent image analyzers of biological microchips. J. Opt. Technol. (Russsian). 65:83-87.

Bavykin, S. G., J. P. Akowski, V. M. Zakhariev, V. E. Barsky, A. N. Perov and A. D. Mirzabekov. 2001. Portable system for microbial sample preparation and oligonucleotide microarray analysis. Appl. Environ. Microbiol., 67: 922-928..

Beyer, W., P. Glöckner, J. Otto, and R. Böhm. 1996. A nested PCR and DNA-

amplification-fingerprinting method for detection and identification of *Bacillus anthracis* in soil samples from former tanneries. Salisbury Medical Bulletin, Special Supplement No. 87:47-49.

Beyer, W., S. Pocivalsek, and R. Böhm. 1999. Polymerase chain reaction-ELISA to detect *Bacillus anthracis* from soil samples — limitations of present published primers. J. Appl. Microbiol. 87:229-236.

Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495-503.

Chee, M., R. Yang, E. Hubbrll, A. Berno, X. C. Huang, D. Stern, J. Winkler, D. J. Lockhart, M. S. Morris, and S. P. A. Fodor. 1996. Accessing genetic information with high-density DNA arrays. Science 274: 610-614.

Daffonchio, D., A. Cherif, and S. Borin. 2000. Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* Group." Appl. Environ. Microbiol. 66:5460-5468.

Delaporte, de M. 1969. Description de *Bacillus medusa* n.sp. C. R. Acad. Sc. Paris 269 (Serie D): 1129-1131.

Drobniewski, F. A. 1993. *Bacillus cereus* and related species, p. 324-338. In Clinical microbiology reviews, Vol. 6. American Society for Microbiology, Washington, D.C..

Ezzell, J. W. Jr., T. G. Abshire, S. F. Little, B. C. Lidgerding, and C. Brown. 1990. Identification of *Bacillus anthracis* by using monoclonal antibody to cell wall galactose-Nacetylglucosamine polysaccharide. J. Clin. Microbiol. 28:223-231.

Fox, A., G. E. Black, K. Fox, and S. Rostovtseva. 1993. Determination of carbohydrate profiles of *Bacillus anthracis* and *Bacillus cereus* including identification of O-methyl methylpentoses by using gas chromatography-mass spectrometry. J. Clin. Microbiol. 31:887-894.

Giffel, M.C., R. R. Beumer, N. Klijn, A. Wagendorp, and F. M. Rombouts. 1997. Discrimination between *Bacillus cereus* and *Bacillus thuringiensis* using specific DNA probes based in variable regions of 16S rRNA. FEMS Microbiol. Lett. 146:47-51.

Gonzalez Jr., J. M., B. J. Brown, and B. C. Carlton. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for β-endotoxin among strains of *B. thuringiensis* and B.cereus. Proc. Natl. Acad. Sci. USA 79:6951-6955.

Guschin, D., G. Yershov, A. Zaslavsky, A. Gemmell, V. Shick, D. Proudnikov, P. Arenkov, and A. Mirzabekov. 1997. Manual manufacturing of oligonucleotide, DNA, and protein microchips. Anal. Biochem. 250:203-211.

Guschin, D. Y., B. K. Mobarry, D. Proudnikov, D. A. Stahl, B. E. Rittmann, and A. D. Mirzabekov. 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. 63: 2397-2402.

Harrell, L. J., G. L. Andersen, and K. H. Wilson. 1995. Genetic variability of *Bacillus anthracis* and related species. J. Clin Microbiol. 33:1847-1850.

Helgason, E., O. A. Økstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A.-B. Kolsto. 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66:2627-2630.

Henderson, I. 1996. Fingerprinting *Bacillus anthracis* strains. Salisbury Medical Bulletin, Special Supplement No. 87:55-58.

Henderson, I., C. J. Duggleby, and P. C. B. Turnbull. 1994. Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. Int. J. Syst. Bacteriol. 44:99-105.

Henderson, I., Y. Dongzheng, and P. C. B. Turnbull. 1995. Differentiation of *Bacillus anthracis* and other *Bacillus cereus* group' bacteria using IS231-derived sequences. FEMS Microbiol. Lett. 128:113-118.

Hutson, R. A., C. J. Duggleby, J. R. Lowe, R. J. Manchee, and P. C. B. Turnbull. 1993. The development and assessment of DNA and oligonucleotide probes for the specific detection of *Bacillus anthracis*. J. Appl. Bacteriol. 75:463-472.

Jackson, P. J., K. K. Hill, M. T. Laker, L. O. Ticknor, and P. Keim. 1999. Genetic comparison of *Bacillus anthracis* and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. J. Appl. Microbiol. 87:263-269.

Kiem, P., A. Kalif, J. Schupp, K. Hill, S. E. Travis, K. Richmond, D. M. Adair, M. Hugh-Jones, C. R. Kuske, and P. Jackson. 1997. Molecular evolution and diveresity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. 179:818-824.

Lander, E. S. 1999. Array of hope. Nature Genet. 21(suppl.):3-4.

Lane, D. J. 1991. 16S/23S rRNA sequencing, p. 115-176. In E. Stackenbrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics, John Wiley & Sons, City, State.

Lechner, S., R. Mayr, K. P. Fransis, B. M. Prub, T. Kaplan, E. Wiebner-Gunkel, G. S. Stewart, and A. B. Scherer. 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Bacteriol. 48:1373-1382.

Lee, M. A., G. Brightwell, D. Leslie, H. Bird, and A. Hamilton. 1999. Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR. J. Appl. Microbiol. 87:218-223.

Liang, X., and D. Yu. 1999. Identification of *Bacillus anthracis* strains in China. J. Appl. Microbiol. 87:200-203.

Longchamp, P., and T. Leighton. 1999. Molecular recognition specificity of *Bacillus anthracis* spore antibodies. J. Appl. Microbiol. 87:246-249.

Nakamura, L. K., and M. A. Jackson. 1995. Clarification of the taxonomy of *Bacillus mycoides*. Int. J. Syst. Bacteriol. 45:46-49.

Nakamura, L. K. 1998. *Bacillus pseudomycoides* sp. nov. Int. Syst. Bacteriol. 48:1031-1034.

Patra, G., P. Sylvestre, V. Ramisse, J. Therasse, and J. L. Guesdon. 1996. DNA fingerprinting of *Bacillus anthracis* strains. Salisbury Medical Bulletin, Special Supplement No. 87:59.

Patra, G., P. Sylvestre, V. Ramisse, J. Therasse, and J.-L. Guedson. 1996. Isolation of a specific chromosomic DNA sequence of *Bacillus anthracis* and its possible use in diagnosis. FEMS Immunol. Med. Microbiol. 15:223-231.

Priest, F. G., D. A. Kaji, Y. B. Rosato, and V. P. Canhos. 1994. Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. Microbiology 140:1015-1022.

Proudnikov, D., E. Timofeev, and A. Mirzabekov. 1998. Immobilization of DNA in polyacrylamide gel for the manufacture of DNA and DNA-oligonucleotide microchips. Anal. Biochem. 259:34-41.

Prub, B.M., K. P. Francis, F. von Stetten, and S. Scherer. 1999. Correlation of 16S ribosomal DNA signature sequences with temperature-dependent growth rates of Mesophilic and Psychrotolerant strains of the *Bacillus cereus* group. J. Bacteriol. 181:2624-2630.

Ramisse, V., G. Patra, H. Garrigue, J.-L. Guesdon, and M. Mock. 1996. Identification and haracterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiol. Lett. 145:9-16.

Ryzhov, V., Y. Hathout, and C. Fenselau. 2000. Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Appl Environ. Microbiol. 66:3828-3834.

Shangkuan, Y.-H., J.-F. Yang, H.-C. Lin, and M.-F. Shaio. 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. J. Appl. Microbiol. 89:452-462.

Sj`stedt, A., U. Eriksson, V. Ramisse, and H. Garrigue. 1996. Detection of the vegetative form of *Bacillus anthracis* in soil by PCR. Salisbury Medical Bulletin, Special Supplement No. 87, 50.

Sneath, P. H. A. 1986. Endospore-forming Gram-positive rods and cocci, p. 1104-1139. In J. G. Holt (ed.), Bergey's Manual of Systematic Bacteriology The Williams & Wilkins Co., Baltimore, MD.

Stahl, D. A. and R. Amann. 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205-248. In E. Stackebrandt and M. Goodfellow (ed.), Sequencing and hybridization techniques in bacterial systematics. John Wiley and Sons, Chichester, England.

Strizhkov, B. N., A. L. Drobyshev, V. M. Mikhailovich, and A. D. Mirzabekov. 2000. PCR amplification on a microarray of gel-immobilized oligonucleotides: detection of bacterial toxin- and drug-resistant genes and their mutations. BioTechniques 29:844-857.

Timofeev, E. N., S. V. Kochetkova, A. D. Mirzabekov and V. L. Florentiev.

1996. Regioselective immobilization of short oligonucleotides to acrylic copolymer gels. Nucl. Acids Res. 24: 3142-3148.

Thorne, C. B. 1985. Genetics of *Bacillus anthracis*, p.56-62. In L. Leive (ed.), Microbiology-1985. American Society for Microbiology, Washington, D.C.

Turnbull, P. C. B. 1999. Definitive identification of *Bacillus anthracis* — a review. J. Appl. Microbiol. 87:237-240.

Turnbull, P. C. B., R. A. Hutson, M. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Kramer, and J. Melling. 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. 72;21-28.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-271.

Wunschel, D., K. F. Fox, G. E. Black, and A. Fox. 1994. Discrimination among the *Bacillus cereus* group, in comparison to B.subtilis, by structural carbohydrate profiles and ribosomal RNA spaser region PCR. Syst. Appl. Microbiol. 17:625-635.

Yamakama, I., D. Nakajama, and O. Ohara. 1996. Identification of sequence motifs causing band compressions on human cDNA sequencing. DNA Research 3:81-86.

Yershov, G., V. Barsky, A. Belgovskiy, Eu. Kirillov, E. Kreindlin, I. Ivanov, S. Parinov, D. Guschin, A. Drobyshev, S. Dubiley, and A. Mirzabekov. 1996. DNA analysis and diagnostics on oligonucleotide microchips. Proc. Natl. Acad. Sci. USA. 93:4913-4918.

Zlatanova, J., and A. D. Mirzabekov. 2001. Gel immobilized microarrays of nucleic acids and proteins. In J. B. Rampal (ed.), Methods in Molecular Biology: DNA Arrays, Methods, and Protocols, in press, Human Press, Inc., Totowa, NJ.